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Nitrogen Metabolism in Plants in the Post-genomic Era

Edited by Christine H. Foyer and Hanma Zhang

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Nitrogen Metabolism in Plants in the Post-genomic Era

Edited by

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CONTENTS

Contributors xiii
Preface xvii

1 Nitrogen Assimilation and its Relevance to Crop Improvement 1
Peter J. Lea and Ben J. Miflin
1.1 Introduction 2
1.2 The assimilation of ammonia 2
1.3 Crop improvement through manipulating genes for nitrogen metabolism 23
1.4 Conclusions 28
Acknowledgements 28
References 28

2 Transcriptional Profiling Approaches for Studying Nitrogen Use Efficiency 41
Malcolm J. Hawkesford and Jonathan R. Howarth
2.1 N-responsive genes 42
2.2 Nitrogen and crop production 46
2.3 Targeting NUtE processes in crop plants 49
2.4 Validating candidate genes by correlating gene expression with complex traits 53
2.5 Prospects 58
Acknowledgements 60
References 60

3 Energetics of Nitrogen Acquisition 63
Arnold J. Bloom
3.1 Availability of nitrogen in the environment 64
3.2 Curiosities 66
3.3 Mineral nitrogen 67
3.4 Plant growth and development 72
3.5 Future of plant nitrogen 75
References 76

4 Transport Systems for NO_3^- and NH_4^+ 83
Mathilde Orsel and Anthony J. Miller
4.1 Nitrogen forms available to plants 83

4.2 Nitrogen transport steps and mechanisms 84
4.3 *Arabidopsis* as a model 86
4.4 Ammonium transporters 86
4.5 Nitrate transporters 89
4.6 Plastid transport 95
4.7 Conclusions and future 96
Acknowledgements 97
References 97

5 Nitric Oxide Synthase-Like Activities in Plants 103
Hideo Yamasaki, Ryuuichi D. Itoh, Josée N. Bouchard, Ata Allah Dghim, Khurshida K. Hossain, Sushma Gurung and Michael F. Cohen

5.1 Introduction 103
5.2 Lifetime of nitric oxide 105
5.3 An overview of NO-dependent signalling systems 106
5.4 Mammalian-type NOS – ghost enzymes in plants 108
5.5 Comparative NO-related signalling 110
5.6 Algal nitric oxide synthesis – an echo from water 113
5.7 Nitric oxide synthase in plant-associated bacteria: its occurrence and functions 114
5.8 Prospects for NO-dependent signal transduction systems in plants 115
5.9 Concluding remarks 117
Acknowledgements 119
References 119

6 Nitrate Reductase and Nitric Oxide 127
Werner M. Kaiser, Elisabeth Planchet and Stefan Rümer

6.1 Introduction 127
6.2 Structure, basic functions and regulation of NR 128
6.3 NR-dependent NO formation *in vivo*, measured as NO emission 130
6.4 NO production by NR *in vitro* 135
6.5 Physiological effects of NR-derived NO 136
6.6 Conclusions and open questions 141
Acknowledgements 141
References 141

7 Nitric Oxide Signalling in Plants: Cross-Talk With Ca^{2+}, Protein Kinases and Reactive Oxygen Species 147
Jéremy Astier, Angélique Besson-Bard, Izabela Wawer, Claire Parent, Sumaira Rasul, Sylvain Jeandroz, James Dat and David Wendehenne

7.1 Basic concepts of NO signalling in animals 148

7.2 NO signalling in plants 152
7.3 Interplays between NO and ROS 158
7.4 Conclusion 162
Acknowledgements 163
References 163

8 Theanine: Its Occurrence and Metabolism in Tea 171
Ning Li and Jacquie de Silva
8.1 Introduction 171
8.2 Physiological benefits of theanine 172
8.3 Chemical properties and characteristics of theanine in tea 173
8.4 Role of theanine in tea 174
8.5 Metabolism of theanine in tea 175
8.6 Theanine synthase 175
8.7 Theanine hydrolase 176
8.8 The site of synthesis and transport of theanine in tea 177
8.9 Other enzymes capable of synthesizing theanine 178
8.10 Nitrogen uptake and transport 179
8.11 Nitrate transporters 180
8.12 Ammonium transporters 182
8.13 Nitrogen assimilation by GS (glutamine synthetase) – GOGAT (glutamate synthase) 184
8.14 Biochemical properties of glutamine synthetase in plants 185
8.15 Gene families of glutamine synthetase 186
8.16 Regulation of plant glutamine synthetase 187
8.17 Glutamate synthase (GOGAT) in plants 189
8.18 Glutamate dehydrogenase in plants 191
8.19 Regulation of theanine – genotypic factors 193
8.20 Regulation of theanine – agronomic factors 194
8.21 Summary 195
Acknowledgements 198
References 198

9 Legume Nitrogen Fixation and Soil Abiotic Stress: From Physiology to Genomics and Beyond 207
Alex J. Valentine, Vagner A. Benedito and Yun Kang
9.1 Introduction 208
9.2 Legume nitrogen fixation under drought stress 213
9.3 Soil acidity 222
9.4 Phosphate deficiency 227
9.5 Legume biology is taking off 232
9.6 Beyond genomics: prospects for legume genetic breeding 233
References 236

10 Metabolomics Approaches to Advance Understanding of Nitrogen Assimilation and Carbon–Nitrogen Interactions 249
Aaron Fait, Agata Sienkiewicz-Porzucek and Alisdair R. Fernie

10.1 Introduction 250
10.2 Methods for analysing the plant metabolome 251
10.3 Uptake and assimilation of nitrate and ammonium 255
10.4 Cross-talk between N and secondary metabolism 258
10.5 Summary 261
References 262

11 Morphological Adaptations of *Arabidopsis* Roots to Nitrogen Supply 269
Hanma Zhang and David J. Pilbeam

11.1 Introduction 269
11.2 N-related morphological adaptations in *Arabidopsis* roots 270
11.3 The developmental context of N-related morphological adaptations in *Arabidopsis* roots 274
11.4 Mechanisms of N-related morphological adaptations in *Arabidopsis* roots 275
11.5 Role of NO_3^- transporters in N-related morphological adaptations 280
11.6 Biological significance of the localized stimulatory effect 281
11.7 Concluding remarks 282
References 283

12 Mitochondrial Redox State, Nitrogen Metabolism and Signalling 287
Christine H. Foyer

12.1 Introduction 288
12.2 The *Nicotiana sylvestris* mitochondrial cytoplasmic male sterile II mutant 289
12.3 Metabolite profiling in CMSII leaves reveals an N-rich phenotype 290
12.4 Mitochondrial redox cycling is a key player in determining the rate of nitrate assimilation 293
12.5 Regulation of pyridine nucleotide metabolism in CMSII leaves 294
12.6 CMSII is an N-sensing/signalling mutant 295
12.7 Regulation of gibberellin metabolism and signalling in the CMSII mutant 297
12.8 Concluding remarks 299
References 299

13 The Utilization of Nitrogen by Plants: A Whole Plant Perspective 305
David J. Pilbeam

13.1 Introduction 306
13.2 Nitrogen and plant growth 306
13.3 Nitrogen, biomass partitioning and yield 312
13.4 Partitioning of nitrogen into metabolites 324
13.5 Acquisition of nitrogen by plants 326
13.6 Plants, nitrogen and environment 335
13.7 Conclusions 340
References 341

Index 353

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PREFACE

A large shift in the activities and dynamics of the field of plant nitrogen metabolism has occurred since the beginning of the millennium. In terms of research in this field, the pre-genomic era was largely characterized by extensive developments in molecular physiology and the identification of transporters and signal transduction cascades, work that was greatly aided by the intensive effort worldwide to sequence different plant genomes. However, after the millennium, we entered the post-genomic era that has concentrated greater effort on elucidating the information concealed within the genome. In the past 10 years, we have witnessed the widespread application of '-omics' (including phenomics, transcriptomics, proteomics and metabolomics) technologies, together with computational biology and bioinformatics in an intensive interdisciplinary cooperation to explore all the facets of nitrogen metabolism from the single cell to whole plant biology and its interactions with the environment. The post-genomic era has facilitated intensive study at these multiple levels of complexity, including a much greater appreciation and understanding of how genome structure supports genome function, but with a deflationary picture of the gene as a structural unit. The recent rise in our understanding of the importance of epigenetic mechanisms of regulation has posed a significant challenge to conventional assumptions about the relationships between genome structure and function, and between genotype and phenotype. An appreciation of recent developments in the field of nitrogen assimilation metabolism is essential for anyone with interests in plant biology, the environment and the needs of agriculture.

The goal of much of the present-day research into plant nitrogen metabolism is to develop and apply new experimental technologies and to provide computer-based methods for coping with and interpreting the vast amounts of -omic data that are being produced and understanding the basic patterns that are being uncovered. The field of plant nitrogen metabolism and improving plant nitrogen use efficiency remains one of the grand challenges of our times. The field continues to require a large basic research aspect, since we are still not close to understanding the interplay of biological systems even at the cellular level. At the same time, the field is faced with a strong demand for immediate solutions, because improving plant nitrogen use efficiency is crucial to future agricultural sustainability and the future economic success of agriculture. The 13 chapters that comprise this volume bring together the expertise and enthusiasm of an international panel of leading researchers to provide a state-of-the-art overview of the field. Topics covered include nitrogen sensing and signalling, uptake and membrane systems, nitric oxide,

primary nitrogen assimilation and C–N balance, and interactions, regulation of root and plant architecture and much more. Together, these chapters provide an up-to-date insight into key issues related to these processes, describing the very latest developments in our understanding of how plants coordinate carbon, nitrate and ammonium assimilation into the organic compounds required for growth on an organ, plant and global scale. The transcription factors that act to integrate environmental nutrient (nitrogen) signals to coordinate primary and secondary metabolism are discussed together with new concepts of cross-talk, transport and signalling, and how such molecular networks influence nitrogen and carbon cycling processes in the environment. The different lines of research and the processes described in these chapters interact in many ways and illustrate how we are starting to disentangle these multiple interactions. These chapters demonstrate the intricacies of this rapidly evolving subject where state-of-the-art post-genomic era technologies are being applied to maximum benefit. We hope that these chapters provide useful, interesting and thought-provoking insights that will prompt further experimentation and breakthroughs in this exciting and expanding field.

Christine H. Foyer and Hanma Zhang

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Chapter 1

NITROGEN ASSIMILATION AND ITS RELEVANCE TO CROP IMPROVEMENT

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Abstract: The majority if not all of the organic nitrogen in plants is derived from the assimilation of ammonia into the amide position of glutamine by the enzyme glutamine synthetase (GS). A second enzyme, glutamate synthase, also known as glutamine:2-oxoglutarate amidotransferase (GOGAT), carries out the transfer of the amide group of glutamine to 2-oxoglutarate to yield two molecules of glutamate and thus completes the assimilation of ammonia into amino acids. This GS/GOGAT pathway of ammonia assimilation is of crucial importance for crop growth and productivity and ultimately animal and human nutrition. Glutamate dehydrogenase (GDH) is now considered to be only involved in glutamate catabolism to form ammonia, an important role in N recycling within the plant. Nitrogen is also often diverted from glutamine to asparagine as a temporary measure during periods of carbohydrate shortage and excess of reduced nitrogen. This diversion requires the action of asparagine synthetase in the anabolic reaction and asparaginase in the catabolic reaction. This chapter describes the properties of these enzymes in the assimilation and reassimilation of nitrogen and in particular the genes that encode them, their complexity and the time and place they are expressed. Plant transformation has allowed the construction of a range of plants with enhanced and decreased activity of several of these enzymes, some of which have shown improved agronomic performance.

Keywords: ammonia assimilation; asparaginase; asparagine synthetase; gene regulation; glutamate dehydrogenase; glutamate synthase; glutamine synthetase; overexpression

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1.1 Introduction

Nitrogen is fundamental to crop productivity, and the increased use of N fertilizer over the past 50 years has led to a massive increase in food production worldwide. This has enabled the support of a vastly increased world population. Whether N is derived from soil reserves, from N fertilizer or from N_2 fixation, it is incorporated into the organic form via the assimilation of ammonia. However, the primary assimilation of ammonia from external inorganic N is only the start of the process. N is released from organic combination as ammonia and reassimilated many times during the movement of N around the plant, from seed reserves, through transport to vegetative organs, to eventual redeposition in a new crop of seeds. There is also a major release and reassimilation of N during the process of photorespiration in C_3 plants. The process of ammonia assimilation is thus of crucial importance to crop growth and productivity. In this chapter, we will review the major enzymes involved in the assimilation of ammonia and the metabolism of asparagine, which is a molecule crucial in the transport and storage of N. We will consider the genes that encode for these enzymes, their complexity and the situations in time and place where they are expressed. Finally, we will review recent progress in research aimed at finding out if there are possibilities to improve plant performance by manipulation of the genes involved in N metabolism.

1.2 The assimilation of ammonia

1.2.1 Glutamine synthetase

The first step in the assimilation of ammonia is the adenosine triphosphate (ATP)-dependent reaction with glutamate to form glutamine, catalysed by glutamine synthetase (GS, EC 6.3.1.2). The ammonia may have been generated by direct primary nitrate assimilation, or from secondary metabolism such as photorespiration (Leegood *et al.*, 1995; Keys, 2006; Forde & Lea, 2007) and the catabolism of amino acids, in particular asparagine (Lea *et al.*, 2007). GS activity is located in both the cytoplasm and chloroplasts/plastids in most but not all higher plants. The GS enzyme proteins can be readily separated by standard chromatographic, localization and Western blotting techniques into cytoplasmic (GS1) and plastidic (GS2) forms. Despite detailed biochemical, molecular and microscopic analysis of conifers, there is no evidence of a plastid GS2 in conifers (Cánovas *et al.*, 2007). It is possible that the localization of GS in the chloroplast in rapidly photorespiring cells (Keys, 2006) may have been a later evolutionary development (Cánovas *et al.*, 2007).

The subunit of cytosolic GS1 has a molecular mass of 38–40 kDa, whilst the plastid GS2 form is larger at 44–45 kDa, and the proteins can be usefully separated by simple sodium dodecyl sulphate polyacrylamide gel electrophoresis

(SDS-PAGE). The quaternary molecular structure of plant GS has proved difficult to establish due to major differences between the eukaryotic and prokaryotic proteins. The bacterial enzyme comprises 12 subunits arranged as two hexameric planar rings. Initially, it was thought that the mammal and plant enzymes were octamers; however, there is now strong crystallographic evidence that both native enzyme proteins exist as decamers, comprising two pentameric rings (Unno *et al.*, 2006; Krajewski *et al.*, 2008). The first crystal structure of a plant GS protein was obtained by Unno *et al.* (2006) using the stable maize GS1a protein. The protein is composed of two face-to-face pentameric rings of identical subunits with a total of ten active sites, each formed between neighbouring subunits within each ring. The first step in the GS reaction is the transfer of the terminal phosphoryl group of ATP to the γ-carboxyl group of glutamate to produce the activated intermediate γ-glutamyl phosphate. In the second step, a bound ammonium ion is deprotonated, forming ammonia, which attacks the carbonyl carbon to form glutamine with the release of phosphate. Unno *et al.* (2006) also studied the binding of the inhibitors methionine sulphoximine and phosphinothricin to the active site of GS as a guide to the development of potential herbicides.

There is now a growing body of evidence that GS activity is regulated by post-translational modification as well as at the level of gene expression. Phosphorylation of cytosolic GS1 and subsequent interaction with 14-3-3 proteins were initially observed by Moorhead *et al.* (1999). Reversible phosphorylation of cytosolic GS1 was then demonstrated during light/dark transitions in the leaves of *Brassica napus* (Finnemann & Schjoerring, 2000). A calcium-dependent kinase-related kinase (CRK3) capable of phosphorylating cytosolic GLN1;1 has been demonstrated in *Arabidopsis thaliana*; both genes *GLN1;1* and *CRK3* were induced in early senescence (Li *et al.*, 2006). Following the discovery of 14-3-3 proteins in the chloroplast, specific binding to GS2 in tobacco was also demonstrated (Riedel *et al.*, 2001). In *Medicago truncatula*, GS2 is regulated by selective phosphorylation of residue Ser 97 and subsequent binding to 14-3-3 proteins, which causes proteolytic breakdown to an inactive product (Lima *et al.*, 2006).

The first steps to isolating and characterizing the genes for GS took place a quarter of a century ago with the isolation of cDNAs for GS from *Phaseolus vulgaris* (Cullimore *et al.*, 1984) and alfalfa cells (Donn *et al.*, 1984). At this stage it was clear that GS was encoded by a multigene family. This was definitively shown for *A. thaliana* when Peterman and Goodman (1991) isolated four different GS cDNAs, indicating the presence of four or possibly five genes. Analysis of the *A. thaliana* genome has identified one *GS2* gene and five *GS1* genes, but only four of which appear to be expressed (Ishiyama *et al.*, 2004b). Subsequent work has shown the presence of multigene families in all species so far studied, generally numbering five or more. These fall into two classes for the two major isoenzyme groups for the enzymes GS1 and GS2. The latter is found in the chloroplast and is encoded by a single gene in most species. Bernard *et al.* (2008) have recently published an analysis of

GS sequences in wheat. Their results show a relatively complex situation with three subclasses of *GS2* genes and some seven GS1 subclasses. This complexity probably reflects the hexaploid nature of wheat, with the three different *GS2* genes coming one each from the different homologous sets of chromosomes. The sequences of the wheat genes, together with those from other species, have been subject to phylogenetic analysis. The results show that the *GS* genes can be divided into a number of related groups (Fig. 1.1). All the *GS2* genes analysed fall into a single clade A, which includes genes from both mono- and dicotyledons. The *GS1* genes from monocotyledons could be divided into three phylogenetic groups (clades B, C and D). In contrast, the allocation of the *GS1* genes from dicotyledons was less certain, and no clear pattern has emerged.

Each of the *GS* genes appears to participate in different metabolic processes, based on where and how they are expressed. With respect to the monocotyledon genes represented in Figure 1.1, the genes in the different clades have different expression patterns. In maize, the putative roles of the five GS1 forms are shown in Figure 1.2 (Martin *et al.*, 2006). Those genes falling in clade B are generally expressed in roots. In some cases they are also expressed throughout the rest of the plant but with a high expression in the root, for example, the genes from rice *OsGS1;2*[1] (Tabuchi *et al.*, 2005), maize *ZmGln1-1* (Hirel *et al.*, 2005) and wheat *TaGSr* (Bernard *et al.*, 2008). Other genes appear to have relatively low expression in the root (*ZmGln1-5*; Sakakibara *et al.*, 1992; Li *et al.*, 1993) and perhaps elsewhere.

The genes falling in clade C are characterized by their presence in the pedicels, spikelets and developing kernels. Thus, *ZmGln1-2* has been shown to be expressed in the developing kernel, pedicel and pericarp and also present in the anthers, glumes and ear husks of maize (Rastogi *et al.*, 1998; Muhitch, 2003). Rice *OsGS1;3* is found specifically in the spikelet (Tabuchi *et al.*, 2005).

The major *GS1* genes expressed in leaves are those that fall in clade D. The maize genes *ZmGln1-3* and *ZmGln1-4* were highly expressed at all leaf ages, although there appeared to be more transcripts of *ZmGln1-4* in older leaves (Hirel *et al.*, 2005). Cytoimmunochemistry and *in situ* hybridization showed that *ZmGln1*-3 is expressed and GS1 protein present in mesophyll cells, whereas *ZmGS1-4* is specifically localized in the bundle sheath cells. *OsGS1;1* is expressed in all organs but with higher expression in leaf blades (Tabuchi *et al.*, 2005). In wheat, *TaGS1a*, *b* and *c* are expressed in the leaves, and their expression increases as the leaf ages, particularly after the onset of anthesis and senescence (Bernard *et al.*, 2008). Several other studies in different species also suggest that GS1 increases in importance in leaves during

[1] Nomenclature for GS genes in the literature is often confusing with *GLN Gln* and *GS* all being used to denote a gene for GS. We have tried to follow the use in the latest papers from the different groups and in Figure 1.1. Mutant versions of the genes are denoted by lowercase letters.

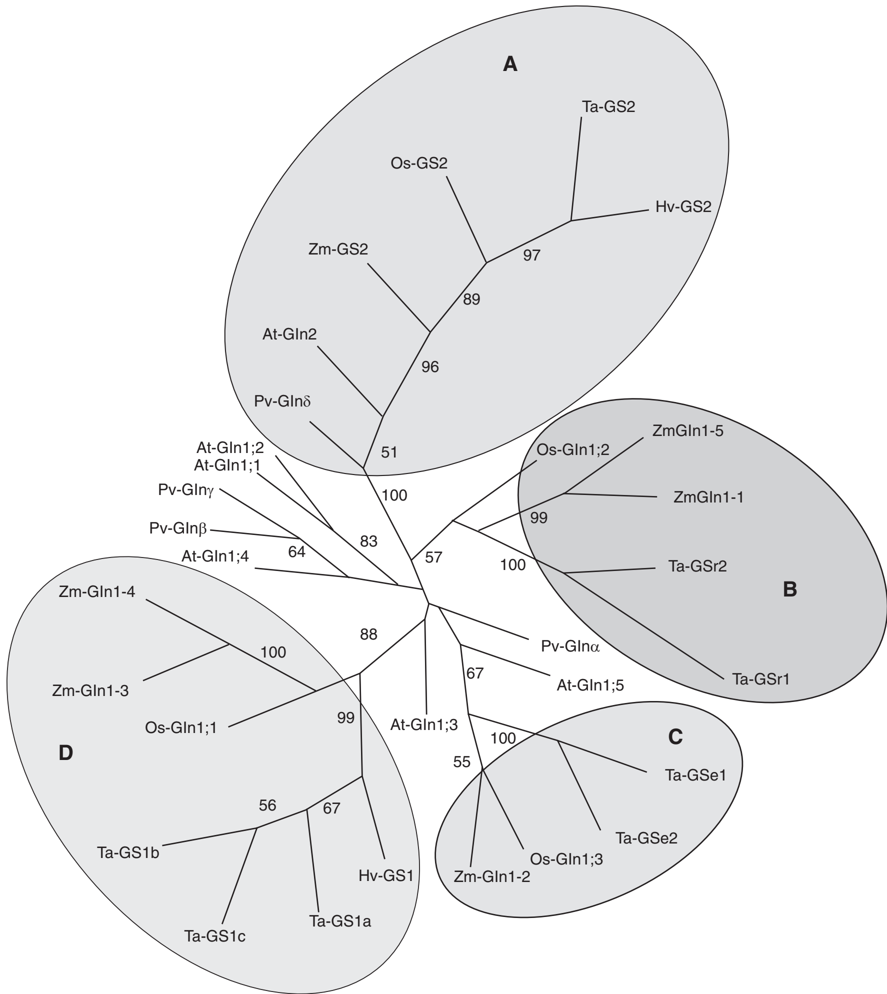


Figure 1.1 Unrooted phylogenetic tree of GS protein sequences from plants. The figure and legend were generously supplied by Dr D. Habash. The figure is reproduced from Bernard *et al.* (2008) by kind permission of Springer Science and Business Media. Analysis was carried out using the PIE interface to the phylogeny inference package (PHYLIP) version 3.5. The proml analysis was used with a JTT model for amino acid change and the tree calculated by the maximum likelihood method. The reliabilities of each branch point were assessed by bootstrap analysis (100 replicates). Bootstrap values are displayed on the tree. Ta-GS2a, AAZ30060; Ta-GSe1, AAR84349; Ta-GSe2, AAR84350; Ta-GSr1, AAR84347; Ta-GSr2, AAR84348; Ta-GS1a, AAZ30057; Ta-GS1b, AAZ30058; Ta-GS1c, AAZ30059; Os-GS2, CAA32462; Os-Gln1;1, CAA32461; Os-Gln1;2, CAA32460; Os-Gln1;3, AAK18848; Zm-GS2, CAA46724; Zm-GS1-1, CAA46719; Zm-GS1-2, CAA46720; Zm-GS1-3, CAA46721; Zm-GS1-4, CAA46722; Zm-GS1-5, CAA46723; Hv-GS2, AA37643; Hv-GS1, CAA48830; At-Gln1;1 (At5g37600), NP_198576; At-Gln1;2 (At1g66200), NP_176794; At-Gln1;3 (At3g17820), NP_188409; At-Gln1;4 (At5g16570), NP_568335; At-Gln1;5 (At1g48470), NP_175280; At-Gln2 (At5g35630), AAB20558; Pv-Gln-δ, AA31234; Pv-Gln-α CAA27632; Pv-Gln-β, CAA27631; Pv-Gln-γ, CAA32759.

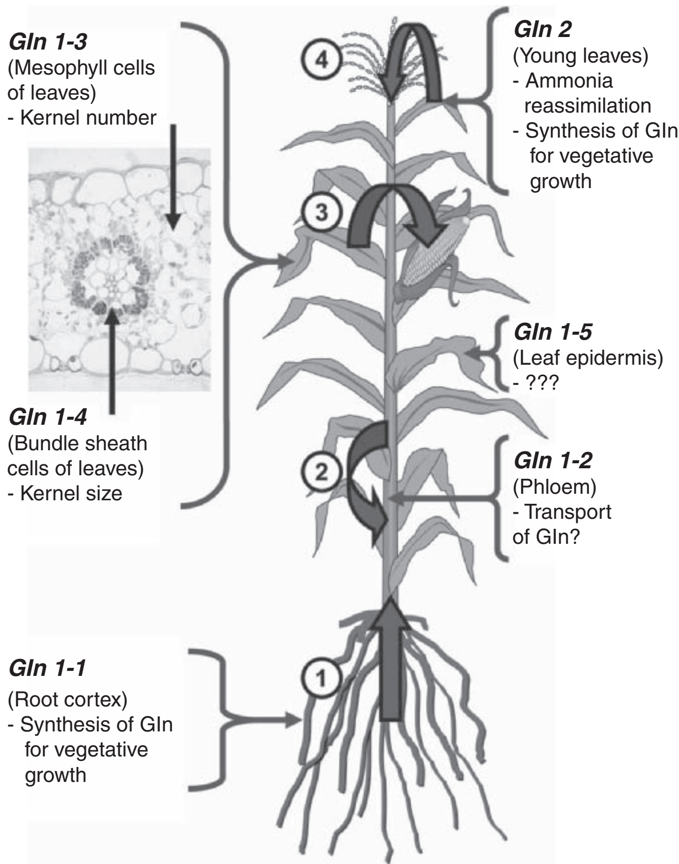


Figure 1.2 Schematic representation depicting the expression and possible function of the GS isoenzymes within a maize plant. The figure and legend were generously supplied by Dr B. Hirel. The copyright ownership of the American Society of Plant Biologists following original publication in Martin *et al*. (2006) is gratefully acknowledged. *Gln1-1* to *Gln1-5* are the five genes encoding cytosolic GS1 and *Gln2* the gene encoding plastidic GS2. Their tissue or cellular localization and their putative function are indicated by small arrows and text. The large arrows indicate the flux of glutamine (Gln) occurring within the plant: 1, from the roots to the shoots (reaction catalysed by GS1-1); 2, in the phloem (reaction catalysed by GS1-2); 3, from the source leaves to the ear (reaction catalysed by GS1-3 and GS1-4); and 4, from the young leaves to the other shoot parts (reaction catalysed by GS2). The function of GS1-5 is unknown, as indicated by question marks.

senescence and GS2 decreases (Brugière *et al.*, 2000; Masclaux-Daubresse *et al.*, 2005; Tabuchi *et al.*, 2007), supporting the role of GS1 in the mobilization of leaf nitrogen into the transport system to developing seeds and other sinks.

Differential expression for the different *GS1* genes has also been described for a number of dicotyledons (Forde & Cullimore, 1989). In *P. vulgaris*, there are three different *GS1* genes termed *Gln-α*, *Gln-β* and *Gln-γ*. *PvGln-γ* is

strongly induced during nodulation, particularly in the infected cells, while *PvGln-β* is preferentially expressed in roots. Studies with promoter fusions of these genes suggest sophisticated differential regulation of the expression of these genes within the cell types of the nodule (Gebhardt *et al.*, 1986; Forde *et al.*, 1989). *PvGln-α* is expressed in the cotyledons and embryonic axis of dry seeds and represents the most abundant GS mRNA in the early days of germination (Swarup *et al.*, 1991). Similar differential expression of these *GS1* genes has been described for soybean, although there are two members of each class of gene; this may reflect the allotetraploid nature of soybean (Morey *et al.*, 2002). In contrast, *Medicago truncculata* has one of the smallest *GS* gene families with only two genes for *GS1* and one for *GS2* (Carvalho & Cullimore, 2003).

GS has also been studied in gymnosperms such as conifers. Interestingly, GS2 does not appear to be present in the conifer family (Garcia-Gutierrez *et al.*, 1998), but there are two *GS1* genes, *GS1a* and *b* (Cánovas *et al.*, 2007). *GS1b* is expressed in vascular tissue, suggesting its role in N transport. *GS1a* is associated with the development of the chloroplast, and its expression is stimulated by light. It is proposed that the physiological role of the enzyme encoded by *GS1a* may be comparable to that of GS2 in angiosperms (Canton *et al.*, 1999).

The *GS* genes exhibit differences in their response to N availability. Studies in *A. thaliana* (Ishiyama *et al.*, 2004b) show that *AtGln1;2* is the only one of four active *GS1* genes that is significantly up-regulated by NH_4^+. In rice, the genes *OsGS1;1* and *OsGS1;2* are reciprocally regulated by NH_4^+ availability. The former accumulates in the surface layers of roots under NH_4^+ limitation, whereas the latter accumulates in the same cell layers when NH_4^+ is sufficient (Ishiyama *et al.*, 2004a). Sakakibara *et al.* (1996) found that, in maize, mRNA for two of the *GS1* genes increased with NH_4^+ nutrition whereas that for the other two decreased. In maize leaves, the major forms (*ZmGln3* and *4*) appear relatively unaffected by N status but *ZmGln1* is much more highly expressed in the leaves under N limitation (Hirel *et al.*, 2005).

The expression of *GS1* genes and the presence of the enzyme differ considerably at the tissue and cellular levels. This has aided the understanding of the different processes in N metabolism in which the enzyme functions (see Fig. 1.2). Sakurai *et al.* (1996) showed that, in rice leaves, GS1 was detected immunologically in the companion cells of large vascular bundles and also in the vascular parenchyma cells of both large and small vascular bundles. This suggested that GS1 was important in the export of leaf N from senescing leaves. Similar results have shown the immunolocalization of GS1 in the vascular tissue of tobacco (Dubois *et al.*, 2003). *In situ* hybridization also showed the transcripts of *NtGln1-5* were localized in the vascular tissues of roots and stems, whereas those of *NtGlt1-3* were found in all root cells and floral organs. Immunolocalization studies in wheat leaves also show GS protein to be present in phloem companion perifascicular sheath cells and in the connections between the mestome sheath cells and the vascular cells in the flag leaf

(Kichey *et al.*, 2005, Bernard *et al.*, 2008). *In situ* localization suggested that this distribution was due to differential expression of different *GS1* genes: *TaGS1* transcripts being expressed in the perifascicular cells and *TaGSr* transcripts being confined to the vascular cells (Bernard *et al.*, 2008).

Mutants of *GS1* genes have been difficult to generate. However, Tabuchi *et al.* (2005) have constructed knockout insertion mutants of *OsGS1;1* in rice. The mutant gene caused the transcription of abnormal mRNA, and the activity of GS1 protein was scarcely detectable in the leaf blades of the homozygous mutants. These mutant plants showed severely retarded growth and poor grain filling under normal N nutrition. Martin *et al.* (2006) studied single and double mutants of maize *GS1* genes caused by Mu insertions into *ZmGln1-3* and *ZmGln1-4.* These genes from rice and maize are homologous and fall in clade D in Figure 1.1. The expression of mRNA was impaired in the single and double maize mutants, which resulted in decreased GS1 protein and activity. At plant maturity, shoot biomass was not modified in either the double or single mutants. There was however a marked effect on grain production; kernel number was reduced in *gln1-3* and kernel size in *gln1-4* mutants, with both characters being decreased in the double mutant. In both species, the mutations could be suppressed by overexpression of the equivalent wild-type *GS1* genes in transgenic plants. These results show that *GS1* genes from clade D are important in grain yield and their function cannot be compensated for by the other *GS1* genes or by *GS2*. The importance of GS1 in anthers and pollen has recently been demonstrated by constructing transgenic tobacco with mutated *GS* genes driven by tapetum and microspore-specific promoters. The plants were male sterile, but this could be reversed by spraying with glutamine (Ribarits *et al.*, 2007).

The gene for *GS2* is highly expressed in the mesophyll of leaves and other photosynthetic tissues from all species analysed. It is a nuclear gene targeted to the chloroplast where the enzyme is located (Lightfoot *et al.*, 1988; Tingey *et al.*, 1988). Its expression is stimulated by light (Lightfoot *et al.*, 1988; Edwards *et al.*, 1990). The isolation of photorespiratory mutants lacking GS2 shows that the major function of GS2 is in the reassimilation of ammonia during photorespiration (Wallsgrove *et al.*, 1987; Blackwell *et al.*, 1988). Taira *et al.* (2004) have proposed that GS2 is dual targeted to both the leaf mitochondria and chloroplasts in *A. thaliana*. This was on the basis of the finding that leaf mitochondria catalysed the transferase activity of GS and could use glycine as the sole source of energy, NH_3 and CO_2 to drive the conversion of ornithine to citrulline. In addition, plants transformed with constructs combining the GS2 promoter with the green fluorescent reporter gene showed fluorescence in the mitochondria. In contrast, Hemon *et al.* (1990) failed to find mitochondrial targeting of GS in transgenic plants unless a specific targeting sequence was added. There are also many reports on the subcellular immunolocalization of GS in several species that fail to show any evidence for GS in the mitochondria (Tobin & Yamaya, 2001; Kichey *et al.*, 2005; Bernard *et al.*, 2008). Bernard *et al.* (2008) also failed to find any evidence for GS2 mRNA outside the

chloroplasts. Wallsgrove *et al.* (1979, 1980) analysed density gradients from barley pea leaf protoplasts but did not observe any GS peak co-migrating with mitochondrial markers, and biochemical studies with isolated mitochondria also failed to provide evidence for a functional GS in mitochondria. Keys *et al.* (1978) examined the ability of purified mitochondria to refix ^{15}N from glycine but found that this required the addition of partially purified GS. Since it is always difficult to confirm the absence of an enzyme, it will be interesting to see if the dual localization of GS2 is corroborated by further experimentation.

In summary, a picture is beginning to emerge of a very complex and sophisticated set of *GS* genes and expression patterns. These reflect the central role of GS in nitrogen metabolism as shown in Figure 1.3 (Miflin & Habash, 2002) in which ammonia is assimilated, released and reassimilated numerous times between the uptake of nitrogen from the environment and its eventual deposition in the protein stores of the seed. However, not all of the regulation

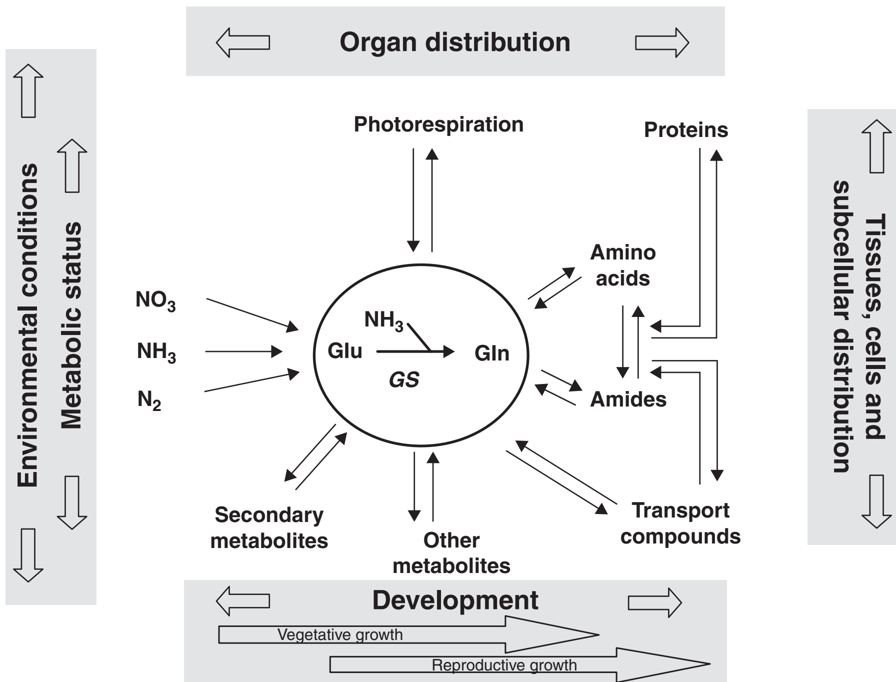


Figure 1.3 The central role of GS in the complex matrix of plant N metabolism. The central scheme encompasses the total role of GS. The boxes around the outside indicate the matrix of various internal and external locations and environments, and stages of plant development, in which GS may be operating. The direction of the flow of N will depend on which part of the matrix is under consideration. Thus, in the developing seed the flux will be from incoming transport compounds towards protein, whilst in the germinating seed the flow will be in the reverse direction. Figure reproduced with permission of Miflin & Habash (2002).

of GS activity is at the transcriptional level (see above and Miflin & Habash, 2002). These regulatory processes are presumably under the influence of further sets of genes which generate yet another layer of genetic complexity of N metabolism.

1.2.2 Glutamate synthase

The second enzyme involved in ammonia assimilation is glutamate synthase, also known as glutamine:2-oxoglutarate amidotransferase (GOGAT). The reaction is a reductant-driven transfer of the amide amino group of glutamine to 2-oxoglutarate to yield two molecules of glutamate. The enzyme in plants is present in two distinct forms, one that uses reduced ferredoxin (Fd) as the electron donor (EC 1.4.7.1) and one that uses reduced nicotinamide adenine dinucleotide (NADH) as the electron donor (EC 1.4.1.14). Both forms of glutamate synthase are localized in plastids, as established by direct measurement of the enzyme activity (Lea & Miflin, 2003) or via immunolocalization studies (Cánovas *et al.*, 2007; Tabuchi *et al.*, 2007). The Fd-dependent enzyme is normally present in high activities in the chloroplasts of photosynthetic tissues, where it is able to utilize light energy directly as a supply of reductant. The NADH-dependent enzyme is located predominantly in non-photosynthesizing cells, where reductant is supplied by the pentose phosphate pathway (Bowsher *et al.*, 2007). The Fd- and NADH-dependent forms of glutamate synthase are expressed differently in separate plant tissues, as will be discussed in a later section.

The bacterial NADPH-glutamate synthase consists of two separate subunits, α (162 kDa) and β (52.3 kDa). The functional protein contains one flavin adenine dinucleotide (FAD), one flavin mononucleotide (FMN) and three different iron–sulphur clusters, one [3Fe-4S] and two [4Fe-4S]. The minimum catalytic structure is the αβ-protomer, but there is now evidence that the active enzyme exists as an $(\alpha\beta)_6$-structure of molecular mass 1.2 MDa (Cottevieille *et al.*, 2008). Data obtained using the enzyme from *Azospirillum brasiliense* (Vanoni *et al.*, 2005; Vanoni & Curti, 2008) have shown that there are six steps in the glutamate synthase reaction, as shown in Figure 1.4. The most interesting is the transfer of the ammonia molecule from the glutaminase site to the synthase site through a 30 Å long intramolecular tunnel, a mechanism now known to be common to all glutamine amidotransferase reactions (Mouilleron & Golinelli-Pimpaneau, 2007).

Initial studies on Fd-glutamate synthase indicated that the protein was monomeric, with experimentally determined molecular masses ranging from 145 to 180 kDa. Calculations of molecular masses from gene sequences provided values of 165.5 kDa for *A. thaliana* and 165.3 kDa for maize, following the removal of plastid transit peptides, leading to the formation of the active enzyme (Lea & Miflin, 2003; Suzuki & Knaff, 2005). A detailed analysis of the crystal structures of Fd-glutamate synthase from the cyanobacterium

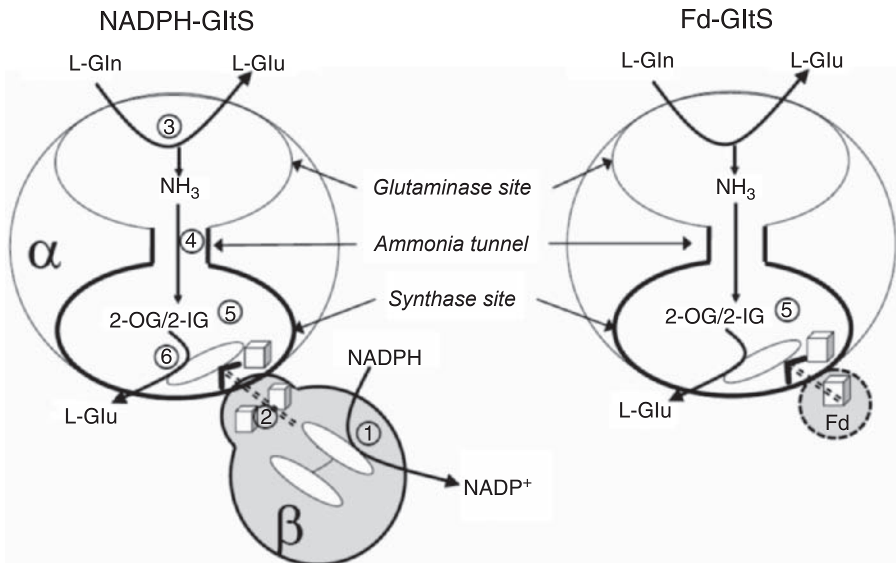


Figure 1.4 The reaction of prokaryotic NADPH-glutamate synthase (GltS), described in a sequence of six steps. The figure and legend were generously supplied by Dr M. Vanoni. The figure is reproduced from Vanoni and Curti (2008) by kind permission of John Wiley & Sons, Inc. Step 1: NADPH binding to the β-subunit and reduction of the FAD co-factor (two linked ovals); Step 2: electron transfer from FAD to FMN (oval) in the synthase domain of the α-subunit through the enzyme iron–sulphur clusters (cubes), namely two low-potential [4Fe-4S] on the β-subunit and a [3Fe-4S] centre on the α-subunit; Step 3: L-glutamine (Gln) binding at the glutaminase site in the PurF-type glutamine amidotransferase domain and hydrolysis with release of the first L-glutamate (Glu) product and ammonia; Step 4: ammonia transfer from the glutaminase to the synthase site through the intramolecular tunnel; Step 5: addition of ammonia to 2-oxoglutarate (2-OG) bound to the synthase site with the formation of the postulated 2-iminoglutarate (2-IG) intermediate; Step 6: reduction of 2-IG to L-glutamate by reduced FMN. In Fd-glutamate synthase (GltS), reduced ferredoxin (Fd) is the electron donor. It has been shown that Fd binds to glutamate synthase protein with a 1:1 stoichiometry in solution.

Synechocystis sp. PCC 6803 has been carried out and has been reviewed in some detail (van den Heuvel *et al.*, 2004; Vanoni *et al.*, 2005). The protein contains one FMN group and one [3Fe-4S] cluster per molecule, but no FAD. Plant Fd-glutamate synthase is similar to the α-subunit of the bacterial enzyme, except that the reducing equivalents are donated following the reversible association of reduced ferredoxin, possibly through a 26-amino acid conserved loop within the FMN domain. It has been proposed that the FMN domain of Fd-glutamate synthase is also involved in the delivery of sulphite to the reaction centre of uridine diphosphate (UDP)-sulphoquinovose synthase (SQD1), thus linking nitrate and sulphate assimilation (Shimojima *et al.*, 2005).

The molecular mass of the NADH-glutamate synthase was originally shown experimentally to be more than 200 kDa. Calculations from the gene sequences indicated that NADH-glutamate synthase in alfalfa nodules has a molecular mass of 229.3 kDa (Gregerson *et al.*, 1993), whilst the enzymes in rice (Goto *et al.*, 1998) and *P. vulgaris* (Blanco *et al.*, 2008) are 236.7 and 241 kDa, respectively, including a plastid transit sequence. The existence of two very similar forms of NADH-glutamate synthase was first demonstrated in *P. vulgaris* nodules (Chen & Cullimore, 1988). All the enzymes studied have been shown to have an FMN binding site, [3Fe-4S] cluster, an NADH binding site and a glutamine amidotransferase site. It is thought that the plant NADH-glutamate synthase protein is a fusion of the prokaryotic α- and β-subunits, with a highly charged hydrophilic connecting region linking the C-terminus of the α-subunit and N-terminus of the β-subunit.

Genes for Fd- and NADH-dependent glutamate synthases have been isolated in a number of plants. The first evidence for *Fd-GOGAT* genes came from mutation studies in *A. thaliana* (Somerville & Ogren, 1980; Somerville, 1986) and barley (Kendall *et al.*, 1986; Blackwell *et al.*, 1988) in which conditional lethal mutants that could not survive under photorespiratory conditions were isolated. Several of these mutants were highly deficient in Fd-glutamate synthase activity. It was thought, based on these mutants, termed *gls* mutants (Somerville 1986), that there was only one Fd-glutamate synthase gene in *A. thaliana*. However, detailed studies by Coschigano *et al.* (1998) showed that there were two genes *GLU1* and *GLU2*. *GLU1* maps to the same local region of the chromosome as *gls1*. This gene is expressed at the highest levels in leaves, and its expression is induced by light. In contrast, *GLU2* is expressed at a low constitutive level in leaves and, preferentially, in roots. It was concluded that *GLU1* functioned in the reassimilation of the NH_4^+ released in photorespiration and some, if not all, of the primary N assimilation in leaves, whereas *GLU2* probably had a function in N assimilation in roots. Similar conclusions have been drawn for the tobacco genes (Feraud *et al.*, 2005).

An *NADH-GOGAT* gene was first isolated from *Medicago sativa* (Vance *et al.*, 1995). There appears to be a single gene, and there is considerable evidence from location and expression studies that it functions in the actively N_2-fixing nodules (Trepp *et al.*, 1999). In contrast, the expression of the *Fd-GOGAT* gene was not detected in nodules. *M. sativa* plants were transformed with antisense constructs of *NADH-GOGAT*, which caused the enzyme activity to be reduced by about 50%. When the plants were grown under N_2-fixing conditions, they were chlorotic, although their ability to fix N_2 did not seem to be impaired. The addition of nitrate restored growth and relieved chlorosis. Perhaps surprisingly, the plants were also male sterile (Schoenbeck *et al.*, 2000). The results suggest that NADH-glutamate synthase functions in N fixation and in developing flowers. Recently, Blanco *et al.* (2008) have isolated two *NADH-GOGAT* genes from *P. vulgaris*. *PvNAPH-GOGATII* was differentially and highly expressed in the developing nodules, while the *PvNADH-GOGATI*

promoter was more active in the meristematic tissues of the root, particularly lateral root primordia.

Rice also has two *NADH-GOGAT* genes (Tabuchi *et al.*, 2007). *OsNADH-GOGAT1* was isolated by Goto *et al.* (1998) and is expressed in developing tissues such as the root tip following the supply of NH_4^+, in the spikelet during the early stages of ripening, and in the premature leaf blades. In contrast, *OsNADH-GOGAT2* is mainly expressed in the mature leaf blade and sheath of the rice plant.

Double-labelling experiments have shown that the Fd- and NADH-glutamate synthase proteins present in the rice root are differentially located and do not overlap. The NADH protein is found in the dermatogen, epidermis and exodermis of the root, while the Fd-protein is found in the meristem, central cylinder and cortex of the roots. Tabuchi *et al.* (2007) have proposed a model in which NH_4^+ is normally assimilated in the outer root zone via NADH-glutamate synthase, and the pathway via the Fd-enzyme only comes into play when there is excess NH_4^+ supply, or NH_4^+ is generated from other reactions locally. It is also thought, mainly based on localization studies (Hayakawa *et al.*, 1994; Tabuchi *et al.*, 2007), that NADH-GOGAT1 is important in developing sink organs in the reutilization of glutamine. The enzyme accumulates in the vascular parenchyma cells and the mestome sheath of developing young leaves, and the dorsal vascular cells of young grains.

A. thaliana appears to have one NADH-glutamate synthase gene (*GLT1*) (Lam *et al.*, 1995). Lancien *et al.* (2002) have probed the function of this gene by constructing a T-DNA insertion in the gene to produce a knock-out mutant, which lacked *GLT1* mRNA and enzyme activity. Under normal growth conditions, the mutant showed little phenotypic difference from the wild type. However, when the plants were grown under 1% CO_2 (i.e. non-photorespiratory conditions), there was a 20% decrease in growth and a 70% decrease in glutamate levels. This shows that the NADH-enzyme has a function in plant metabolism, different from the Fd-glutamate synthases, which may be important in aspects of root glutamate synthesis and remobilization of N. This is possibly similar to the role proposed above for the rice enzyme.

As for GS, the genetic control of glutamate synthase activity is complex in which a number of genes with complementary roles have been described for several important crop species. Again, these different genes are likely to have specific and non-redundant roles in the different pathways of N metabolism, indicated in Figure 1.3.

1.2.3 Glutamate dehydrogenase

The two enzymes, involved in glutamate synthesis discussed previously, catalyse irreversible reactions. A third enzyme, glutamate dehydrogenase (GDH; EC 1.4.1.2), catalyses a reversible amination/deamination reaction, which could lead to either the synthesis or catabolism of glutamate. GDH extracted from most plant species can be readily separated into seven

isoenzymic forms following native gel electrophoresis (Thurman *et al.*, 1965; Loulakakis & Roubelakis-Angelakis, 1996). The reason for this is that GDH comprises two distinct subunits α (43 kDa) and β (42.5 kDa) that are able to assemble, apparently at random into enzymatically active hexamers. The relative proportion of the α- and β- subunits, and hence the isoenzyme pattern observed, varies with plant organ and nitrogen source (Loulakakis & Roubelakis-Angelakis, 1991; Turano *et al.*, 1997). Mutant and antisense lines deficient in GDH have been obtained in maize (Magalhaes *et al.*, 1990; Pryor 1990), *A. thaliana* (Melo-Oliveira *et al.*, 1996; Myashita & Good, 2008) and tobacco (Fontaine *et al.*, 2006), which confirm the roles of the α- and β-subunits in the production of the seven banded isoenzyme patterns.

By far the major proportion of higher plant GDH can only utilize NAD(H) as a coenzyme. This form of GDH is located in the mitochondria, particularly within the phloem companion cells of shoots (Paczek *et al.*, 2002; Tercé-Laforgue *et al.*, 2004a; Fontaine *et al.*, 2006). Immunolocalization studies in *Nicotiana tabacum*, wheat and vine (Paczek *et al.*, 2002; Dubois *et al.*, 2003; Tercé-Laforgue *et al.*, 2004b; Kichey *et al.*, 2005) suggest that under certain circumstances, for example senescing leaves, flower receptacles and in epidermal root tip cells, the enzyme is also localized in the cytoplasm. There have also been reports of an NADP-GDH present in the chloroplasts of higher plants (Lea & Thurman, 1972; Turano *et al.*, 1997), although the activity is low and difficult to measure (Miyashita & Good, 2008). However, high ammonium-inducible NADP-GDH activity has been fully characterized in green algae such as *Chlorella sorokiniana* (Bascomb *et al.*, 1987; Cock *et al.*, 1991; Jaspard, 2006), where it is located in the chloroplasts.

Two cDNAs and/or genes for the NAD enzyme have been cloned from a number of species corresponding to the two polypeptides (Purnell *et al.*, 2005). The two genes in *A. thaliana* have been termed *GDH1* and *GDH2* (Melo-Oliveira *et al.*, 1996; Turano *et al.*, 1997) and code for the β- and α-subunits, respectively (Melo-Oliveira *et al.*, 1996; Purnell *et al.*, 2005; Fontaine *et al.*, 2006; Miyashita & Good, 2008). Analysis of the *A. thaliana* sequence database by Purnell *et al.* (2005) identified the presence of a second DNA sequence that could code for a β-subunit. This sequence (*At3g03910*) was reported as being expressed using microarray analysis (Yamada *et al.*, 2003). However, enzyme analysis of *A. thaliana gdh1* mutants did not show any evidence for the expression of a β-subunit (Melo-Oliveira *et al.*, 1996; Fontaine *et al.*, 2006; Miyashita & Good, 2008), which would be expected if *At3g03910* led to the expression of a functional protein. The conclusion is that there is probably only one functional gene for the β-subunit in *A. thaliana*.

Genes for GDH have been isolated from *Nicotiana* species. *Nicotiana plumbaginifolia* has two genes *GDHA* and *GDHB* (Ficarelli *et al.*, 1999; Restivo, 2004) that code for the α- and β-subunits, respectively. Masclaux-Daubresse *et al.* (2002) isolated a *GDH1* gene from *N. tabacum* coding for a β-subunit that was 95% sequence identical with the *GDHB* gene from *N. plumbaginifolia*. Purnell *et al.* (2005) isolated two partial cDNA clones for GDH that were 98%

homologous to the *N. plumbaginifolia* genes *GDHA* and *GDHB*, respectively. The sequence homology between the *GDH1* reported by Masclaux-Daubresse *et al.* (2002) and the cDNA for *GDHB* reported by Purnell *et al.* (2005) was 89%, leading the latter authors to suggest that there were two genes for the β-subunit of GDH in *N. tabacum*. Genes for the α-subunit have been identified in maize (Sakakibara *et al.*, 1995) and *Vitis vinifera* (Syntichaki *et al.*, 1996).

The expression of the α- and β-subunit genes differs throughout the plant and in response to different external stimuli. The *A. thaliana* gene *GDH2* was expressed in all *A. thaliana* tissues tested, whereas transcripts of *GDH1* were not found in roots (Turano *et al.*, 1997) except at a very low level (Melo-Oliveira *et al.*, 1996). The two genes also respond differently to the supply of nitrogen and carbohydrate. Thus, while *AtGDH1* is the prevalent form in rosette leaves in the light, *AtGDH2* is preferentially expressed in those leaves when plants are kept in the dark, with or without sucrose (Turano *et al.*, 1997; Miyashita & Good, 2008); in addition, Melo-Oliveira *et al.* (1996) have found that the expression of *GDH1* is suppressed by sucrose. In tobacco callus cultures, *GDHA* mRNA was more abundant when the cultures were grown in NH_4^+, whereas the *GDHB* mRNA increased when the carbon source was removed. The two genes also responded differently to NaCl stress, with the *GDHA* transcript being increased by NaCl and the *GDHB* decreased (Restivo, 2004). Similar results on the effect of salt stress on the expression of the *GDHA* gene were found in *N. tabacum* plants, and the expression of the equivalent *V. vinifera* gene was increased by NH_4^+ (Skopelitis *et al.*, 2006). In summary, the two *GDH* genes are differentially regulated with respect to different tissues and organs of the plant and in respect of different conditions of N and C metabolism, stress and senescence. Comparison of the transcript and enzyme levels of GDH suggests that the two are not always correlated and thus there may be transcriptional and translational levels of control (Restivo, 2004; Purnell *et al.*, 2005). There is also evidence of compensatory mechanisms in which one gene can compensate for the absence of the other (Fontaine *et al.*, 2006).

There is some evidence for the presence of *NADP-GDH* genes in higher plants. The gene sequence from *Chlorella sorokiana* (Cock *et al.*, 1991) was used to probe various *Triticum* species (Boisson *et al.*, 2005), and a number of sequences that could possibly code for NADPH-GDH were identified. Purnell *et al.* (2005) also reported the identification of a clone in the rice and *A. thaliana* genome databases that could code for the enzyme.

The role of GDH has been the subject of much debate since the discovery of the GS/GOGAT pathway in plants (see Lea & Miflin, 1974, 2003; Miflin & Lea, 1976; Miflin & Habash, 2002). The discovery of the genes and the production of mutants of GDH have provided materials to test the role of GDH in plants. Pryor (1990) constructed a homozygous recessive maize mutant lacking *GDH1*. The phenotype of the plant is altered compared to the wild type in that it is more cold-sensitive and it also has a lower shoot-to-root ratio. This mutant was used by Magalhaes *et al.* (1990) in a kinetic study of

^{15}N assimilation. It was found that the mutant had a lower flux of ^{15}N via glutamate than the wild type, which could suggest that GDH has a role in ammonia assimilation. However, the flux of ^{15}N through NH_4^+ to glutamate in both mutant and wild type was abolished by application of methionine sulphoximine, a potent inhibitor of GS. This strongly suggests that GDH was not carrying out ammonia assimilation. Studies by Stewart *et al.* (1995) with the same mutant appear to confirm this conclusion and suggest that the GDH encoded by *GDH1* is functioning in the direction of oxidative deamination. However, some reservation should be placed on these maize studies since the two genotypes are not isogenic. Melo-Oliveira *et al.* (1996) constructed a *gdh1* mutant of *A. thaliana* that appeared to have an impairment of root growth and some shoot chlorosis when grown under high N nutrition. Although the authors state that this suggests that GDH plays a non-redundant role in ammonia assimilation, they provided no biochemical evidence for this and the results could equally be explained by GDH playing an important role in the deamination of glutamate to produce keto acids, as suggested by Miflin and Habash (2002). It is interesting that in these studies no *gdh2* mutants were recovered. However, Alonso *et al*. (2003) generated a genome-wide series of TDNA insertions in *A. thaliana* from which two GDH mutants were identified, *gdh1* (SALK_042736) *and gdh2* (SALK_0102711), in which the production of, respectively, either the α- or β-subunit was suppressed. Fontaine *et al.* (2006) did not report any phenotypic effect of either of the single mutants on plant growth. In part, this may have been because they observed strong compensatory effects on the production of GDH encoded by the gene that was not mutated. Recently, Miyashita and Good (2008), using the same mutant stocks, have constructed the double mutant *gdh1/gdh2* and studied this as well as the single mutants separately. The double mutant has no detectable GDH protein or activity. They grow and reproduce under normal growth conditions without a visible phenotype. The mutations have no effect on the ability of the plants to grow on inorganic N, but the *gdh2* and *gdh1/gdh2* mutants are impaired in their ability to grow on glutamate as an N source. The double mutant also exhibited marked leaf necrosis under prolonged dark treatments. The results are compatible with the hypothesis that GDH plays no part in N assimilation but suggest there are a number of conditions in which the deamination activity may be important for plant function.

1.2.4 Asparagine synthetase

Although not strictly on the direct route of ammonia assimilation, nitrogen is often diverted from glutamine to asparagine as a temporary measure during periods of carbohydrate shortage and reduced nitrogen excess. The periods when asparagine can accumulate include seed germination, senescence, nitrogen transport and storage and a wide range of stress conditions and nutrient deficiencies (Sieciechowicz *et al.*, 1988; Lea *et al.*, 2007; Lehmann & Ratajczak, 2008). Recently, there has been a considerable revival of interest in

the accumulation of asparagine in plant foodstuffs due to its capacity to combine with sugars in the Maillard reaction during cooking to form acrylamide, a highly toxic compound (Mottram *et al.*, 2002; Halford *et al.*, 2007).

The major route of asparagine synthesis involves the initial assimilation of ammonia to the amide position of glutamine as described above, followed by the transfer to form the amide position of asparagine (Ta *et al.*, 1986; Rhodes *et al.*, 1989; Lea *et al.*, 2007). The enzyme asparagine synthetase (AS; EC 6.3.5.4) catalyses the ATP-dependent transfer of the amide amino group of glutamine to a molecule of aspartate to generate glutamate and asparagine:

$$\text{Glutamine} + \text{aspartate} + \text{ATP} \rightarrow \text{glutamate} + \text{asparagine} + \text{AMP} + \text{PPi}$$

It has also been proposed that the AS enzyme can use ammonia directly as a substrate under certain circumstances (Oaks & Ross, 1984). Direct evidence was presented by Masclaux-Daubresse *et al.* (2006), who showed that, in tobacco leaves, ^{15}N-labelled ammonia was incorporated into the amide position of asparagine in the dark in the presence of azaserine, whilst assimilation into glutamate was totally inhibited. Carvalho *et al.* (2003) demonstrated an inverse relationship between the gene expression of GS and AS in *M. truncatula* nodules and proposed that ammonia-dependent AS could operate in the direction of asparagine formation when the activity of GS was low. However, this suggestion was later modified, when it was considered that a shortage in the supply of aspartate would limit asparagine synthesis (Barsch *et al.*, 2006).

Measuring the expected levels of activity of the enzyme AS in plant tissues has proved a difficult task. On many occasions authors have reported very low or zero levels of activity, although the particular plant tissue has been shown to be synthesizing asparagine at high rates (Lea *et al.*, 2007). AS has been assayed and studied to a limited extent in germinating seedlings, nitrogen-fixing root nodules and maize roots. The enzymes isolated from these different sources exhibited similar properties. The purification to homogeneity of AS from alfalfa root nodules has been reported (Shi *et al.*, 1997), but no details of the methods used, or the kinetic properties, were provided. Gálvez-Valdivieso *et al.* (2005) were able to express both genes encoding AS isolated from *P. vulgaris* in *Escherichia coli*. The *PvAS2*-encoded protein was used to raise the antibody that recognized both *P. vulgaris* gene products with a molecular mass of 66 kDa. AS protein was detected in mature roots, senescing leaves and only very early in the development of the root nodules of *P. vulgaris* (Gálvez-Valdivieso *et al.*, 2005). Again, unfortunately, no attempt was made to study the properties of the enzyme protein.

Genes encoding plant AS (*AS1* and *AS2*) were initially isolated from peas by Tsai and Coruzzi (1990, 1991), and two encoded proteins of molecular mass 66.3 and 65.6 kDa were shown to be highly homologous with the human enzyme. Northern blot analysis indicated that the expression of both genes was repressed by light in the leaves, but that in the roots *AS2* was expressed constitutively and only *AS1* was repressed by light. The repression of the genes encoding AS by light and stimulation in the dark agreed with the early

work showing that asparagine accumulation was stimulated by darkness (Lea *et al.*, 2007).

An asparagine synthetase cDNA clone was isolated from Asparagus spears that encoded a 66.5 kDa protein that was 81% identical to the AS1 from pea (Davies & King, 1993). It was proposed that the induction of asparagus AS mRNA was stimulated by a rapid reduction in the soluble sugar content. Later analysis of the promoter of the asparagus gene identified a potential carbohydrate-responsive element at –410 to –401 bp relative to the translation initiation ATG, with sequence identity to a rice α-amylase carbohydrate-responsive element (Winichayakul *et al.*, 2004a). Further studies confirmed that low carbohydrate but not darkness acted as the signal for the induction of the promoter of asparagus AS (Winichayakul *et al.*, 2004b), probably through the involvement of hexokinase.

This simple story of carbohydrate regulation became somewhat complicated when it was found that there were three genes encoding AS in *A. thaliana* (Lam *et al.*, 1994, 1998), which appeared to be regulated in a totally different manner. Expression of *ASN1* was stimulated when plants were placed in the dark and dramatically repressed following exposure to light for only 2 hours, whilst sucrose to some extent could substitute for light. In contrast, the expression of *ASN2* was induced during the same period and further stimulated for another 16 hours in the light, and again sucrose could substitute for light. Even more interestingly, the expression of *ASN1* was stimulated by the amino acids asparagine and glutamine and glutamate, whilst *ASN2* was repressed by the same amino acids. Expression of the *ASN*3 gene was not detected in any of the organs examined. Further studies in *A. thaliana* by Thum *et al.* (2003) indicated that light was able to override sucrose in the regulation of *ASN1*, whilst sucrose was able to override light as the major regulator of *ASN2*. There was also evidence that blue and red light had differential effects on the expression of the AS genes. Recently, Hanson *et al.* (2008) have shown that the transcription factor bZIP11 is involved in the regulation of the sugar-repressible *ASN1* gene of *A. thaliana* through a G-box element present in the promoter.

The influence of carbon on the regulation of AS gene expression was further studied by Silvente *et al.* (2008) in the tropical legume *P. vulgaris*, which is a ureide rather than an asparagine transporter. The AS gene *PvNAS2* was highly expressed in the roots and to a lesser extent in the outer cortical cells of root nodules during the early stages of nitrogen fixation. The addition of sugars induced *PvNAS2* expression and asparagine production. A model was proposed in which when the rate of nitrogen fixation is high, the sugar content is low and AS is down-regulated, leading to the diversion of nitrogen from glutamine to purine and ureide synthesis. Hexokinase was again postulated as being involved in the sugar-sensing mechanism in *P. vulgaris* (Silvente *et al.*, 2008).

Three distinct genes encoding asparagine synthetase have also been identified in sunflower (*Helianthus annuus*) (Herrera-Rodriguez *et al.*, 2002, 2004, 2006). *HAS1* and *HAS1.1* were shown to be light-repressed genes whose

transcripts accumulated to high levels in darkness. Light regulated the genes by means of two different mechanisms, a direct one, via phytochrome, and an indirect one, stimulating photosynthetic CO_2 assimilation and the production of carbon metabolites such as sucrose. The third AS gene of sunflower, *HAS2*, was regulated by light and carbon in an opposite manner to that of *HAS1* and *HAS1.1. HAS2* had a high level of constitutive expression and was stimulated by light and sucrose. *HAS1* and *HAS1.1* expressions were dependent on the presence of a nitrogen source, while *HAS2* transcripts were still found in N-starved plants. High ammonium levels induced all three AS genes and partially reverted the sucrose repression of *HAS1* and *HAS1.1* (Herrera-Rodriguez *et al.*, 2004).

To investigate the involvement of asparagine and AS genes in the main nitrogen mobilization processes in sunflower, the expression of *HAS1*, *HAS1.1* and *HAS2* genes, as well as the synthesis of asparagine and other nitrogen and carbon metabolites, was studied during germination and natural senescence of cotyledons and leaves (Herrera-Rodriguez *et al.*, 2006). *HAS2* was expressed early in germination, and there was a correlation between the AS transcript level and asparagine accumulation in the sunflower tissues. Throughout leaf senescence, all three genes were expressed, during which time there was a reduction in sucrose content. In a later study on sunflower, it was shown that the expression of *HAS1* and *HAS1.1* particularly in the roots was induced by salt stress, osmotic stress and heavy metal stress and reduced by heat stress, whilst that of *HAS2* was not affected (Herrera-Rodriguez *et al.*, 2007).

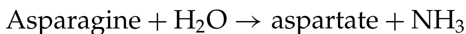
Genes encoding AS have now been isolated from a wide range of species (Lea *et al.*, 2007). Although there is considerable variation between plants in the exact mechanisms involved in the regulation of the expression of AS, there is an overall consensus. The expression of one gene (often that which is most highly expressed) is induced by a reduction in soluble carbohydrate supply and, in some cases, darkness, while a second gene may be stimulated by carbohydrate and light. An increased supply of reduced nitrogen, either as ammonium or amino acids, induces the expression of AS genes.

Analysis of the amino acid sequences of plant AS shows that there is a high percentage of identity with the *E. coli* AS-B protein, which has been studied in some detail due to its potential for the screening of chemotherapeutic compounds (Richards & Kilberg, 2006). In the *Pinus sylvestris* sequence, binding sites for glutamine, aspartate and an adenosine monophosphate (AMP) anchoring site have been identified (Cañas *et al.*, 2006). Phylogenetic trees of the plant amino acid sequences have been constructed by a number of researchers and compared to bacteria, fungi and animals (Shi *et al.*, 1997; Osuna *et al.*, 2001; Møller *et al.*, 2003). In the most recent study of the *P. sylvestris* enzyme, the plant sequences were clustered in two main groups: (1) the sequences close to *A. thaliana* AS1 and (2) those grouped with *A. thaliana* AS2 and AS3. The legume sequences were located in the AS1 cluster, whilst the monocot sequences were in the AS2/3 group (Cañas *et al.*, 2006).

1.2.5 Asparaginase

As indicated previously, asparagine synthesis and accumulation occur under specific conditions, often those of stress and/or carbohydrate deficiency. Once the situation changes, there is a need to liberate the nitrogen from asparagine for the synthesis of other amino acids. There are two established pathways of asparagine catabolism in higher plants, and these have been considered in detail by Joy (1988) and Sieciechowicz *et al.* (1988). Asparagine can be transaminated, particularly in leaves, to yield oxosuccinamic acid, which may then be reduced to hydroxysuccinamic acid and subsequently deamidated to yield malate. It is likely that asparagine is metabolized through the above route as part of the photorespiratory nitrogen cycle (Keys, 2006), but that the majority of the nitrogen is continuously recycled and that there is little net catabolism of asparagine.

The major route of asparagine catabolism is via the enzyme asparaginase (EC 3.5.1.1), which catalyses the hydrolysis of asparagine to yield aspartate and ammonia. The ammonia is subsequently reassimilated by the GS/glutamate synthase enzymes as described above:



$$\text{Asparagine} + H_2O \rightarrow \text{aspartate} + NH_3$$

The assay of asparaginase has also proved difficult in higher plants, with some plant sources providing extracts with high rates of activity and others low or zero. A full description of the early setbacks has been provided by Sieciechowicz *et al.* (1988) and Lea *et al.* (2007).

It was the detailed investigation by Atkins *et al.* (1975), who first gave the important indication that particularly high asparaginase activity could be detected during the development of *L. albus* cotyledons inside the pod. Subsequently, a number of workers confirmed the presence of high activities of asparaginase in legume seeds during the maturation process (Lea *et al.*, 1978; Murray & Kennedy, 1980; Chang & Farnden, 1981). Sodek *et al.* (1980) then described an asparaginase in both the testa and maturing cotyledons of peas that was totally dependent on the presence of potassium (K^+) ions. The asparaginase in developing soybean cotyledons was also shown to be K^+-dependent (Gomes & Sodek, 1984; Tonin & Sodek, 1990). A K^+-dependent asparaginase has been studied in some detail in pea leaves, where the enzyme is only functional in the light when there is sufficient ATP and reducing power to fuel the GS/glutamate synthase reactions (Sieciechowicz *et al.*, 1988).

It was originally demonstrated that the native K^+-independent asparaginase from *Lupinus polyphyllus* seeds was a dimer of molecular mass of 71–72 kDa with subunits of 35–38 kDa (Lea *et al.*, 1978; Sodek & Lea, 1993). Lough *et al.* (1992a) went on to show that when asparaginase was purified from *Lupinus arboreus* seeds, although the native molecular mass was 75 kDa, three polypeptides in the range 14–19 kDa were present following SDS gel electrophoresis. At the time, the reason for the complex number of subunits was

not clear. Sodek *et al.* (1980) reported a native molecular mass of 68 kDa for the pea cotyledon K^+-dependent enzyme, whilst the enzyme protein in pea leaves had a lower native molecular mass of 58 kDa (Sieciechowicz *et al.*, 1988).

A cDNA clone encoding a K^+-dependent asparaginase was isolated from *L. arboreus*. This encoded a 32.8 kDa protein, which appeared to be only expressed at a specific time during seed maturation, coinciding with high enzyme activity. Somewhat surprisingly the gene was not expressed in roots, which had also been shown to have high asparaginase activity (Chang & Farnden, 1981; Lough *et al.*, 1992b). Dickson *et al.* (1992) isolated a genomic sequence encoding asparaginase from *Lupinus angustifolius* that contained four exons and three introns. The 5′-flanking region contained sequences associated with nodule-specific and seed-specific expressions.

The promoter of the asparaginase gene isolated by Dickson *et al.* (1992) was ligated to a GUS reporter gene and transformed into tobacco plants (Grant & Bevan, 1994). GUS activity was found mainly in the developing tissues of mature plants such as apical meristems, expanding leaves, inflorescences and seeds of tobacco. The chimaeric gene was also used to investigate transient expression in lupins. As might be expected from earlier enzyme measurements, transient GUS expression was detected in the developing pods, seed testas and cotyledons.

A major breakthrough in our understanding of the molecular structure of plant asparaginases was obtained when Hejazi *et al.* (2002) were able to express the *A. thaliana* gene in *E. coli*. The purified asparaginase protein was shown to comprise peptides of approximately 35, 24 and 12 kDa, following SDS-PAGE. The authors proposed that the two smaller peptides were the result of proteolytic cleavage and that the native protein, rather than being a dimer, was in fact an $(\alpha\beta)_2$-tetramer. Analysis of the substrate specificity of the recombinant *A. thaliana* protein showed that the enzyme could use a range of β-aspartyl peptides as substrates, with β-aspartyl-phenylalanine and β-aspartyl-alanine having V_{max} values close to that of asparagine.

Borek *et al.* (2004) expressed a gene encoding the *Lupinus luteus* K^+-independent asparaginase (Borek *et al.*, 1999) in *E. coli*. The recombinant native enzyme had a molecular mass of 75 kDa, but the peptide underwent an autoproteolytic cleavage, leading to the formation of two subunits of 23 kDa (α-subunit) and 14 kDa (β-subunit), confirming the existence of the $(\alpha\beta)_2$-tetramer. This cleavage gives rise to an N-terminal nucleophilic threonine residue on the β-subunit. Phylogenetic analysis of N-terminal nucleophilic hydrolases indicated that the amino acid sequences of the plant asparaginases from *A. thaliana*, *L. luteus*, barley, rice and soybean fell in a group with bacterial enzymes that also had isoaspartyl peptidase activity. Although asparagine was a substrate for the recombinant *L. luteus* enzyme with a K_m of 4.8 mM, the surprising result was that β-aspartyl-leucine was a substrate with over four times the V_{max} and a K_m of only 0.14 mM (Borek *et al.*, 2004).

Michalska *et al.* (2006a, 2006b, 2008) crystallized the K^+-independent asparaginase from *L. luteus* and carried out a detailed analysis of the structure. The protein exhibited an αββα-fold typical of N-terminal nucleophilic hydrolases. Each of the two active sites of the $(\alpha\beta)_2$-heterotetrameric protein is located in a deep cleft between the β-sheets, near the nucleophilic threonine-193 residue, which is liberated in the autocatalytic event at the N-terminus of the β-subunit. A comparison of the active sites of the *L. luteus* asparaginase and the *E. coli* EcAIII enzyme showed a high degree of conservation of the residues participating in substrate/product binding and of all other residues forming important hydrogen bonds within the catalytic pocket. Some evidence was provided as to how the active site could accept both asparagine and β-aspartyl peptides.

The availability of the complete sequence of the *A. thaliana* genome allowed Bruneau *et al.* (2006) to isolate a second gene encoding an asparaginase enzyme that was dependent on K^+ for full activity. The K^+-dependent enzyme had 55% identity with the K^+-independent form, indicating that they belong to two evolutionarily distinct subfamilies of plant asparaginases, as revealed by phylogenetic analysis. However, the two enzyme proteins had remarkably similar structures, the K^+-dependent enzyme having α-subunits of 22.7 kDa and β-subunits of 13.6 kDa. In addition, there were conserved autoproteolytic pentapeptide cleavage sites, commencing with the catalytic threonine nucleophile, as determined by ESI-MS analysis. The K^+-dependent enzyme in *A. thaliana* had a lower K_m and much higher V_{max} than the K^+-independent form, indicating an 80-fold higher catalytic efficiency with asparagine. The K^+-dependent enzyme was unable to use β-aspartyl dipeptides as substrates, demonstrating a clear difference between the enzyme and the K^+-independent enzyme (Bruneau *et al.*, 2006).

The steady-state mRNA levels of the two asparaginase genes in *A. thaliana* were determined by quantitative reverse transcription polymerase chain reaction (RT-PCR) in various tissues during development. As expected, the expression of both genes was associated with sink tissues, and was highest in flowers, siliques, flower buds and leaves. The two genes showed largely overlapping patterns of developmental expression, but in all the tissues examined, the transcript levels of the K^+-dependent enzyme were lower than those of the K^+-independent enzyme. Microarray analysis showed that the K^+-dependent enzyme was highly expressed in stamens and mature pollen of *A. thaliana* (Schmid *et al.*, 2005). Cho *et al.* (2007) isolated a cDNA (*GmASP1*) from soybean that encoded a K^+-dependent enzyme expressed in leaves. The expression of *GmASP1* mRNA was induced by low temperature and salt stress, but not by heat shock or drought stress.

As part of a study of both the synthesis and catabolism of asparagine in germinating seedlings of *P. sylvestris*, Cañas *et al.* (2007) isolated the *PsASPG* gene encoding a K^+-dependent asparaginase. The gene encoded a 39.7 kDa protein, which following proteolysis could be split into subunits of 27 and 12 kDa. However, this process required an accessory protein and was not

an autocatalytic reaction as had been shown with other plant asparaginases. During the early stages of seed germination in *P. sylvestris*, the hypocotyl is the major site of asparagine production and the AS gene *PsAS1* is highly expressed (Cañas *et al.*, 2006). However, at later stages when the hypocotyl develops a more vascularized structure, the expression of *PsAS1* decreases and that of *PsASPG* increases, probably indicating a major shift from asparagine production to consumption (Cañas *et al.*, 2007).

Bruneau *et al.* (2006) suggested that as the spatial patterns of the expression of the two asparaginase genes were largely overlapping, that the two enzymes had redundant functions. As mutants and knockout lines are not currently available, it is not possible to test this hypothesis. However, the key question is why should plants have one form of an asparaginase, which apparently has a greater activity and a higher affinity for isoaspartyl peptides? One possible reason is due to the frequently occurring conversion of asparagine to isoaspartyl residues in mature proteins. This is a dangerous modification, as it causes a structural change that may significantly alter the three-dimensional structure of the protein, leading to a change of activity, degradation or aggregation. Proteins with isoaspartyl residues can be degraded by proteolytic enzymes, but among the products there will be β-aspartyl peptides containing N-terminal isoaspartyl residues which require specialized hydrolytic enzymes (Shimizu *et al.*, 2005). Borek *et al.* (2004) proposed that isoaspartyl peptidase activity could be particularly important in seeds that have to retain their ability to grow for a very long time. During the storage period, the seed proteins can undergo modification and isoaspartyl peptidase activity is necessary to destroy the altered proteins and to allow only the healthy seeds to germinate.

1.3 Crop improvement through manipulating genes for nitrogen metabolism

The metabolism of nitrogen by crops is of prime importance to crop productivity. It also has a strong impact on the environment due to the need to fertilize many crops with N fertilizer or the release of N from crop residues, particularly legumes. Crop improvement over the last century has progressed tremendously through selection-based improvement grounded in a knowledge of genetics. We are now at the stage where direction-based crop improvement is becoming a reality, by the use of either transformation techniques or genetic marker-based selection. Both approaches with respect to N metabolism are discussed below.

1.3.1 Identification of markers of N metabolism important in crop performance

DNA marker technology such as RFLPs (restriction fragment length polymorphisms) has allowed the locations of genes important in determining

polygenic traits – the so-called quantitative trait loci (QTL) – to be identified. It is implicit in the technique that there is allelic variation between genotypes at these loci so that differences can be measured. The more favourable alleles at the different loci can then be assembled in the same plant to potentially provide improved genotypes. For more detailed discussion of the technology, see reviews by Tanksley (1993) and Collard and Mackill (2008).

Several approaches in different crops have been used to try to identify loci of consequence to nitrogen metabolism and also to see if such loci are also important in aspects of crop performance. The advantage of this approach is that it can deal with traits controlled by several genes and that the nature of the genes does not have to be predetermined so that new interesting loci and eventually genes can be identified. Thus, determination of loci important in the amount of GS activity in a plant could identify regulatory, as well as structural, loci for the enzyme. There are a number of challenges inherent in the approach. Defining the trait to be measured is not simple; for example, crop improvement in relation to N metabolism can be considered in terms of yield, of seed protein content (high or low according to the use of the crop) for a given yield or nitrogen use efficiency (NUE). Deciding on a measurement of NUE is itself complex, and Good *et al.* (2004) review a number of definitions that have been used. Once the traits to be measured have been defined, then it is critical that they are measured accurately. This can often involve complex field experiments. Finally, the genetic crosses and resultant population of offspring have to be established. In this it is crucial that the parents are different in the traits to be measured, as only loci at which there are allelic differences have the potential to be revealed as important in determining the trait under consideration.

Obara *et al.* (2001) mapped QTL in a cross between varieties of japonica and indica strains of rice that affected the content of cytoplasmic GS1 and NADH-glutamate synthase. Seven QTL regions were detected for GS1 and six for NADH-glutamate synthase protein content. Some of these mapped to regions where a structural gene was located, for example, the structural gene for NADH-glutamate synthase on chromosome 1. Other loci were linked to both GS1 content and earlier leaf senescence. Interestingly, the region containing a structural gene for GS1 (*OsGS1;1*) on chromosome 2 was associated with a QTL for spikelet weight. The importance of this locus in crop performance was confirmed by studying near isogenic lines in which a 50 cM segment of the indica chromosome 2 was transferred into the japonica background (Obara *et al.*, 2004).

Hirel *et al.* (2001) analysed leaves of inbred maize lines that had been assessed for their agronomic performance for certain physiological traits associated with N metabolism. QTL for various agronomic and physiological traits were located on the genetic map of maize. Coincidences of QTL for yield and its components with genes encoding GS1 and the corresponding enzyme activity were observed. Gallais and Hirel (2004) extended these findings and recognized three chromosome regions where there were coincidences in QTL

for yield, N-remobilization, GS activity and a structural gene for GS1. They proposed that the GS structural gene on chromosome 5 (*ZmGln1-4*) could be a candidate gene to explain variation in N use efficiency. This is in line with the studies on the maize *gln1-3* and *gln1-4* mutants (Martin *et al.*, 2006). Further discussion of these issues in maize is given in Hirel *et al.* (2007).

Mickelson *et al.* (2003) reported on the identification of QTL in barley, associated with nitrogen uptake, storage and remobilization in flag leaves relative to QTL for grain protein and certain development traits. Among their results, they found that alleles associated with inefficient N remobilization were associated with depressed yield, but that the most prominent QTL for grain protein did not co-locate with the QTL for N remobilization. Unfortunately, they did not have the genes for GS marked on their map. Habash *et al.* (2007) have reported on a similar study in wheat in which they determined QTL for 21 traits related to growth, yield and leaf N assimilation during grain fill. One cluster of QTL for GS activity co-localized with the *TaGS2* gene on chromosome 2A and another mapped to a region containing the *TaGSr* gene on chromosome 4A. This study also provided a wealth of detail as to the interrelationships between different physiological traits and yield components.

This brief review, of the use of QTL mapping in trying to identify the important N metabolism genes, indicates that results have been obtained suggesting possible links between GS structural genes and yield components. In this regard, they are consistent with the mutant studies in maize and rice, identifying the importance of *GS1* genes from clade D in relation to yield components. They suggest that selection of the favourable alleles at these QTL could lead to crop improvement. The studies also identify other chromosomal regions, without structural genes for major enzymes of N metabolism, important in contributing to the control of aspects of the N economy of plants. Future studies hold the promise of identifying and isolating genes important in regulatory aspects of N metabolism that contribute to crop improvement.

1.3.2 Effect of transforming plants with N metabolism genes on crop performance

A number of species of plants have been transformed with genes important in N metabolism, and the effect of these transformed genes has been measured on a range of characters, including NUE (see Andrews *et al.*, 2004; Good *et al.*, 2004; Lea & Azevedo, 2007, for further discussion). In the main, the genes have been sourced from plants other than the host and a number of different promoters used to drive the expression of the genes. The majority of these studies have been carried out with *GS* genes.

Early studies were done with tobacco, mainly using the CaMV35S promoter linked to a *GS1* gene, and led to increased production of GS in the transformed plants (Eckes *et al.*, 1989). In many cases, the transformed plants had relatively high enhancement of GS1 transcripts with a lesser enhancement of GS1 protein and only subtle effects on GS activity (Habash *et al.*,

2001; Ortega *et al.*, 2001; Fei *et al.*, 2003). There is also evidence of compensation between GS1 and GS2 enzyme levels in transgenic plants. For example, wheat lines transformed with the *GS1* gene *PvGln-α* under the control of the Rubisco small subunit promoter had more GS1 and less GS2 enzyme activity in the flag leaves during grain filling (Habash *et al.*, 2001).

Effects on the growth characteristics of the transgenic plants have been reported. Fuentes *et al.* (2001) found that T1 generation tobacco plants, transformed with *CaMV35S:GS1* constructs, were similar to the wild type under high N nutrition but were greener and had higher shoot and root dry weight than the controls in low N. In contrast, Oliveira *et al.* (2002) found that similar tobacco transformants grew better under both N-sufficient and N-limiting conditions. In general, the effects were observed in younger plants and later in development they were harder to establish. In part, this might be because transformed plants appeared to mature earlier (Vincent *et al.*, 1997). In many of the studies, the effects varied between different transformed lines in the same experiment (Fei *et al.*, 2003, 2006).

Martin *et al.* (2006) transformed maize with a *GLN1-3* gene, driven by a constitutive CsVMV promoter, and the resulting plants had higher activities of GS1 and an increase in grain yield and number. Some wheat lines transformed with the *GS1* gene *PvGln-α*, under the control of the Rubisco small subunit promoter, also showed increased grain yield, although this was due to increased grain weight (Habash *et al.*, 2001). In contrast, overexpression of *OsGS1;1* in rice did not lead to any yield benefits (Tabuchi *et al.*, 2007).

Most experiments on annual plants have been done with single plants grown in pots. This may not equate to what may occur in a monoculture field, where the plants compete with each other. Thus, it is not clear that enhancing the overall level of GS1 through transformation will lead to improved crop productivity in annual crops. This may be because of the complex and subtle way in which GS is located and regulated during the life cycle of a plant. Success may need to await the development of more sophisticated transformation strategies, particularly the use of specific promoters, that take this complex system into account. More consistent results have been obtained with transgenic clones of hybrid poplar (Gallardo *et al.*, 1999). Clones expressing a *GS1a* gene from the conifer *P. sylvestris*, under the control of the CaMV35S promoter, have been shown to have higher vegetative growth with enhanced nitrogen assimilation efficiency, higher net photosynthetic rates, higher rates of photorespiration and enhanced resistance to water stress (see Kirby *et al.*, 2006, for a review). Many of these benefits have been sustained in a 3-year field test (Jing *et al.*, 2004).

Kozaki and Takeba (1996) constructed tobacco plants with higher and lower expression of a GS2 transgene and reported that the higher level of GS2 increased their capacity for photorespiration and enhanced their resistance to high light intensity. Later experiments with rice (Hoshida *et al.*, 2000) showed that the GS2 transgenics again had higher photorespiratory capacity and had increased tolerance of salt stress. However, studies with barley and oil seed

rape, in which the activity of GS2 had been reduced, provided variable results as to the effect on the rate of photorespiratory ammonia release (Leegood *et al.*, 1995; Husted *et al.*, 2002). Migge *et al.* (2000) also used a *GS2* gene fused to a Rubisco small subunit promoter and reported increased growth of the transformed tobacco seedlings. However, older plants did not differ in size, but the transformants were developmentally more advanced, reflecting similar effects observed in GS1 transformants. Although the young plants had markedly higher level of GS2 transcripts, the enzyme activities were only about twofold higher and by the time plants were mature there was no difference in GS activity between the transformants and wild-type plants. Taken together with results from GS1 transgenics, the data suggest that there are a series of post-transcriptional and translational controls of GS enzyme activity that operate to modify the effects of the overexpression of the GS transgene.

Overexpression of a chimeric *NADH-GOGAT* gene in rice produced some transgenic lines that had enhanced glutamate synthase activity and which appeared to have higher spikelet weights, supporting the importance of NADH-glutamate synthase in grain filling (Yamaya *et al.*, 2002). Tobacco plants transformed with *CaMV35S:NADH-GOGAT* had higher levels of enzyme activity, particularly in the roots, and higher C and N content and shoot dry weight as the plants were beginning to flower (Chichkova *et al.*, 2001).

Tobacco plants have been transformed with an *E. coli NADPH-GDHA* gene, fused to a CaMV35S promoter, and subjected to a number of phenotypic tests including field trials. It has been reported that these plants are more resistant to methionine sulphoximine than the wild type and had more biomass in the greenhouse and the field (Ameziane *et al.*, 2000). Maize was also transformed with the *E. coli* gene under the control of an ubiquitin promoter (Lightfoot *et al.*, 2007). These plants had increased levels of GDH, although those with the highest enzyme activity were infertile. The fertile transformants were selfed, back-crossed and crossed with B73 to produce hybrids. Each of these was field tested. Plants with the *E. coli GDHA* gene had increased biomass but only in seasons and locations where there were water deficits. The results suggested that the *EcGDHA* gene may improve plant performance under arid conditions.

Brears *et al.* (1993) transformed tobacco with a pea gene for AS with the CaMV35S promoter. The plants accumulated more asparagine, but there was no statistically significant increase in growth. Later, Lam *et al.* (2003) found that *A. thaliana* plants transformed with a similar construct produced seeds that had an improved N status, in that the seeds had more soluble and total protein and the young germinating seedlings grew better under N-limiting conditions. Brears *et al.* (1993) also transformed plants with a gene modified to be able to use NH_4^+ rather than glutamine but the plants grew more slowly. Bellucci *et al.* (2004) used an NH_4^+-dependent AS gene from *E. coli* to transform *L. corniculatus*. Those plants that expressed the gene were also characterized by reduced growth and premature flowering. In contrast,

some lines of oilseed rape plants containing a similar construct showed an advantage in higher seed N yield under high N supply (Seiffert *et al.*, 2004).

Good *et al.* (2007) transformed canola (*Brassica napus*) with an alanine dehydrogenase cDNA under the control of a root-specific promoter. The transgenic plants had increased biomass and seed yield, both in the laboratory and the field, under low N conditions, whereas no differences were observed under high N. The results suggested that the transgenics required 40% less applied nitrogen fertilizer to achieve yields similar to the wild type.

1.4 Conclusions

The research reviewed here has tended to confirm the generalities of the established biochemical pathways for N assimilation and cycling around the plant. What has changed radically in recent years is the understanding of the complexity of the genetics and controls of enzyme activity. Nowhere is this more evident than for cytosolic glutamine synthetase, which is encoded by multiple genes with different expression patterns and subject to multiple levels of control. The availability of genes for the major enzymes has increased the potential for constructing mutants lacking a specific gene and thereby enabled the testing of the function of the gene product. This has led to the confirmation of the general role of GS in ammonia assimilation and of the inability of GDH to perform the same function. It has also allowed the roles of some of the individual genes encoding GS1 to be evaluated.

The availability of genes and methods of plant transformation have allowed the creation of a variety of plants with enhanced activity of several enzymes of N metabolism. In a few cases the transformed plants have shown improved agronomic performance. More sustained improvements probably await the second series of transgenics with more targeted transformations. The use of genetic markers has also indicated that improvement of crops with respect to N metabolism could be possible. In particular, regions of the chromosome containing *GS1* genes have been linked with crop improvement. Both of these developments show promise for the future.

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Chapter 2

TRANSCRIPTIONAL PROFILING APPROACHES FOR STUDYING NITROGEN USE EFFICIENCY

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Abstract: Nitrogen use efficiency comprises multiple interacting subtraits. In agronomic terms, the two major components are nitrogen uptake efficiency and nitrogen utilization efficiency, the former dominated by root acquisition processes and the latter describing harvestable yield as function of the nitrogen taken up. The complexity of both of these processes is ideally tackled using genomic approaches including transcriptomics. Profiling gene expression in relation to nitrogen supply and with the consideration of germplasm variation is a route to identifying genes underpinning key traits involved in the efficient use of nitrogen. Substantial progress has been made on identifying nitrogen-responsive genes in model plant systems; however, a challenge remains to incorporate these technologies into crop improvement programmes. An approach is detailed where field agronomic performance is related to specific gene expression in canopy tissues, which is indicative of the efficiency of nitrogen remobilization processes from the canopy to the grain in wheat. Identified genes include transcription factors likely to be involved in controlling expression of multiple genes encoding the various components in the complex pathways contributing to the traits.

Keywords: crop production; microarray; nitrogen use efficiency; transcriptomics; wheat

Transcriptome profiling provides information on levels of expression of all genes in a genome in the sampled tissue at a specific moment in time and under defined conditions, limited only by the extent of the array platform and the resolution of the tissue sampling. Systematic approaches including

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Table 2.1 Definitions of nitrogen use efficiency

Parameter	Abbreviation	Definition
Nitrogen uptake efficiency	NUpE	Nitrogen taken up into the crop (above ground)/nitrogen available in soil
Nitrogen utilization efficiency	NUtE	Grain yield/nitrogen taken up into the crop (above-ground tissue)
Nitrogen use efficiency	NUE	NUpE × NUtE = yield/available nitrogen
Nitrogen harvest index	NHI	Nitrogen in grain/nitrogen in the whole crop at harvest

omics-based technologies such as transcriptome profiling are ideally suited for the study of a complex trait, such as nitrogen use efficiency (NUE, for definition see Table 2.1). The availability of high throughput transcriptomics platforms has facilitated studies on nitrogen nutrition-responsive gene expression in *Arabidopsis* and in some crop species, including the identification of nitrate-inducible genes, representing characterized pathways, novel genes and genes of unknown function. Recently, the advent of a range of transcriptome platforms for rice and wheat, including spotted microarrays consisting of cDNAs (Zhu *et al.*, 2003; Lu *et al.*, 2005; Lian *et al.*, 2006) and photolithographically produced arrays of *in situ* generated short oligonucleotides of the type typified by the Affymetrix arrays (Wilson *et al.*, 2004; Wan *et al.*, 2008), has enabled dissections of processes relating to NUE. The available technologies for transcriptome analysis specifically in wheat have been reviewed critically (Leader, 2005).

2.1 N-responsive genes

Nitrogen is essential for the formation, growth and functioning of all plant tissues throughout development, and availability determines plant growth via the provision of amino acids for protein synthesis. Additionally, nitrogen-containing compounds, for example nitrate, can act as signals of nitrogen availability and plant nutritional status, resulting in a cascade of responses involving carbon, nitrogen and sulphur nutrition, as well as carbon allocation, root growth and whole plant development (Forde, 2002). Transcriptomics has emphasized the complexity of these responses, simultaneously facilitating the identification of the gene-encoding components of the pathways involved, and interacting control elements such as transcription factors.

Transcriptomics has been widely applied to identify nitrogen-regulated genes and to characterize responses to nitrogen availability, particularly in *Arabidopsis*. Whilst the processes involved in NUE in crop plants will have both developmental and architectural components, as well as involving many

aspects of metabolism, it is likely that basic responses to cellular nitrogen availability form the basis of many of the key processes involved in efficient nitrogen uptake and utilization. Prior to application of microarrays, several genes had been identified as being nitrogen-regulated, notably nitrate reductase and nitrate transporters. The availability of genetic resources for *Arabidopsis* allowed an early application of transcriptomic tools, and this has provided a useful model to gain insights into fundamental aspects of plant nutrition in relation to regulation of gene expression and cellular metabolic pathways. Initial microarray studies with *Arabidopsis* indicated that the 40 most responsive genes (on a cDNA array of 5524 genes) included most of the known nitrate-regulated genes including a nitrate transporter (NRT1), nitrate reductase and glutamine synthetase, and additionally many novel genes including regulatory proteins (Wang *et al.*, 2000). The typical pattern was for a transient increase in expression in response to low nitrate and a sustained increase in expression in the presence of high nitrate. Other genes were repressed by nitrate, including AMT1;1, an ammonium transporter, and ANR1, the MADS box gene (Zhang & Forde, 1998), known to have a role in control of lateral root proliferation. In a study using a microarray consisting specifically of nutrient-regulated cDNAs, 115 cDNAs (out of 1280) were shown to be nitrate-regulated in tomato (Wang *et al.*, 2001). Both these studies hinted at the complex changes in patterns of gene expression resulting from major perturbation of the nitrogen supply, a not surprising result given the key role of nitrogen in plant development.

Application of increasingly sophisticated microarrays, encompassing the whole genome and refinements of experimental design, increased both the confidence of identified gene sets and the numbers of responsive genes (Wang *et al.*, 2003; Gutierrez *et al.*, 2007a). Over 100 rapidly responding genes were identified using the ATH1 Affymetrix microarray chip. In addition to known genes such as nitrate transporters, nitrate reductase and pentose phosphate pathway components, further pathways were shown to be nitrogen-responsive including genes encoding enzymes involved in glycolysis (glucose-6-phosphate isomerase and phosphoglycerate mutase), in trehalose-6-phosphate metabolism, in iron transport and metabolism and in sulphate uptake. Responses in roots were more extensive (1176 genes) than in the leaves (183 genes); however, the interacting controls with carbon metabolism were clearly identified. The responsiveness of the root transcriptome indicates the complex network of metabolism in the root involved in nitrogen acquisition and processing and is suggestive that processes pertaining to the root and NUE should not be overlooked. An interesting observation was that only some members of large gene families were nitrogen-responsive (Wang *et al.*, 2003), with the clear implication that transcriptome studies need to possess this resolution. Transcriptome studies using a nitrate reductase mutant, unable to grow on nitrate as a source of nitrogen, confirmed that nitrate specifically, rather than downstream reduced nitrogen-containing compounds, is an important signal controlling expression of many

(595) genes (Wang *et al.*, 2004). Interestingly, starch mobilization was not induced by nitrate but responds to ammonium addition (Wang *et al.*, 2004); this leaf-specific response is an adaptation ensuring that carbon and nitrogen metabolism is coordinated at a point after nitrate uptake and is therefore more closely related to biosynthetic demands. An alternate approach using methionine sulphoximine to inhibit glutamine synthetase and prevent ammonium assimilation identified many genes responding to downstream reduced nitrogen-containing compounds rather than nitrate as previously assumed (Gutierez *et al.*, 2008). This study highlighted the involvement of the circadian clock in controlling nitrogen assimilation as well as a possible influence of nitrogen nutrition on clock functioning (Gutierez *et al.*, 2008). This regulatory interaction is an adaptive response to coordinate cellular metabolism during changing diurnal conditions, and is another factor which must be taken into account when considering NUE in crops.

Superimposition of transcriptome data on metabolic pathways facilitates visualization of responses to nitrogen (Scheible *et al.*, 2004). Data from ATH1 arrays indicated that the familiar reprogramming of metabolism was apparent in response to limiting nitrogen, which was rapidly reversed upon resupply. A clear consequence of this is the plasticity of cellular responses to transient supply, that is the ability of a plant to maximize utilization of available nitrogen, for example responding to limited nitrogen by switching metabolism to a 'standby' mode. Additionally in this study, a high-throughput polymerase chain reaction (PCR) platform was used to identify a large number of candidate regulatory elements, which however require further analysis to confirm or reject their involvement in the control of the nitrogen response pathways (Scheible *et al.*, 2004). Of the approximately 1800 transcription factors represented in this high-throughput platform, 93 showed marked changes in expression in response to nitrogen nutrition. These transcription factors are key targets for biotechnological application, as they are responsible for coordinating multiple genes and have the potential to regulate a whole network, whereas other single gene manipulations are often compensated for or restricted by the same gene network.

A further level of sophistication combines transcriptome data with profiles of metabolites (the metabolome), thus providing a link of gene expression through to actual metabolism (Hirai *et al.*, 2004). The additional information provided by metabolomics allows for network analysis and the construction interacting maps of genes and metabolites, and has been previously used extensively in relation to sulphur metabolism and more recently for the analysis of nitrogen responses. In this study it was estimated that about half the *Arabidopsis* transcriptome responds to carbon, nitrogen or C/N interactions (Hirai *et al.*, 2004; Gutierrez *et al.*, 2007b). A multiple network model was constructed including 6176 genes and 1459 metabolites giving rise to 230 900 interactions. The overriding conclusion is that C/N sensing is critical and therefore the interaction between these resources and the pathways involved in harnessing them is critical for plant's well-being. This has direct application

to the understanding of NUE in crops which are defined as yield, essentially carbon fixation, as a function of nitrogen input.

Limitations of bulk transcriptome analysis of whole plant or even selected organs were highlighted in a study in which cell sorting was used on root cells. A several-fold increase in sensitivity allowed identification of previously unidentified nitrogen-responsive genes and allowed the implication a specific transcriptional regulatory mechanism, controlling lateral root growth involving microRNAs (Gifford *et al.*, 2008). This study highlights the care required, interpreting whole tissue analysis, and emphasizes the need to be as selective as possible when analysing specific responses.

The transcriptome changes observed for nitrogen limitation differ substantially from those proposed for nitrate induction (Peng *et al.*, 2007). Genes involved in protein degradation through autophagy and ubiquitin–proteosome pathways as well as amino acid catabolism are up-regulated together with genes encoding components of anthocyanin and phenylpropanoid pathways. Genes encoding components of the photosynthetic and pathways involving biosynthesis of nitrogen-containing metabolites and macromolecules including proteins and nucleotides are all down-regulated. Furthermore, a ubiquitin ligase gene (*NLA, nitrogen limitation adaptation*) facilitates these responses to nitrogen limitation, at least in *Arabidopsis* (Peng *et al.*, 2007); *nla* mutants fail to modify gene expression and show early senescence with an inability to induce anthocyanin and increased lignification (Peng *et al.*, 2008). The overall pattern is clearly one to limit growth and to channel available nitrogen to essential processes only, including synthesis of stress-protective compounds. Although not explicit in this study, the consequent recycling of nitrogen is most logically targeted at generative tissue and seed production.

As already indicated, specificities in architecture and developmental patterns of crop plants may be important contributors to specific responses determining efficient nitrogen use in these species; therefore, transcriptome analyses will be a key component in studies of NUE for the respective crop. Transcript profiling studies to identify rapidly nitrogen-regulated genes have been performed in young rice seedlings (Lian *et al.*, 2006). In this study seedlings were transferred to low nitrogen-containing media and the transcriptome expression profiles were determined after 20 minutes, 1 hour and 2 hours. The array platform provided analysis of 10 422 unique genes, and whilst no significant differential expression was detected in the leaves during this short-term stress, a total of 471 expressed sequence tags (ESTs) were detected as responsive to nitrogen in the roots, including genes involved in energy metabolism and known generic stress-related genes, as well as signal transduction pathways and transcription factors. Clearly, with such a large number of regulated genes, including many of unknown function, the challenge is to prioritize the key genes as well as to establish their precise roles and contribution to nitrogen response pathways. A limitation to this study, and others examining root responses, is that these are invariably performed in hydroponic systems to facilitate manipulation of nitrogen supply and ease

of root harvesting. It is highly likely that in a field situation, with likely heterogeneous nitrogen supply and a multitude of other interacting factors, even more genes will be involved. The complexity of NUE in such a real situation is huge. In terms of crop improvement, multiple genes may need to be targeted or alternatively network hubs or transcriptional global regulators targeted.

2.2 Nitrogen and crop production

Nitrogen is a major determinant of yield and quality in crops. However, nitrogen fertilizer application is costly and can have negative environmental impacts. Excess nitrogen can affect air quality via nitrous oxide and ammonia emissions, and highly nitrogenous run-off water from arable land may cause ecological problems including eutrophication of inland lakes, rivers and coastal waters. Production and use of nitrogen fertilizers have large carbon footprints and nitrous oxide emissions, which occur due to overfertilization and are extremely damaging greenhouse gases. Improving the efficiency of use of applied fertilizer is essential for the success of meeting yield demand whilst minimizing environmental impacts. Both management practices and improved germplasm will contribute to efficient nitrogen fertilizer use and facilitate reduced inputs and reduced wastage.

Efficient use of nitrogen may be defined in many ways, and the definitions referred to in this chapter are listed in Table 2.1. The overall measure of efficiency, NUE, that is the grain yield per unit of nitrogen available to the crop, is the product of two separate and independent traits: nitrogen uptake efficiency (NUpE) and nitrogen utilization efficiency (NUtE). Another measure, the nitrogen harvest index (NHI), is the portion of nitrogen from total nitrogen taken up which partitions into the harvested organ (in wheat, the grain), and clearly, a greater NHI will be reflected in lower losses of applied fertilizer. These measurements are usually made on a whole crop basis rather than per individual plant. In addition, such criteria take little account of critical quality issues such as protein content (hence, nitrogen content) of the grain, an important contributor to grain quality. In terms of a single plant, these measurements, along with the key biochemical processes that contribute to them, are illustrated in Figure 2.1. NUpE is the ability to take up and store nitrogen from the soil and will depend on root architecture, longevity and functioning. NUtE is the efficiency of carbon fixation for the nitrogen taken up and includes processes involving photosynthesis, canopy formation, activity and longevity, as well as nutrient remobilization from all tissues to grain during seed filling. NHI is specifically dependent on senescence of the canopy tissues and the ability to transport resources (in this case, nitrogen) to the grain. In all cases, multiple processes controlled by multiple genetic loci contribute to these traits, and whilst many are undoubtedly related to the processes involved in the various, mostly *Arabidopsis*, transcriptome studies published to date, there are also likely to be many crop-specific critical pathways and

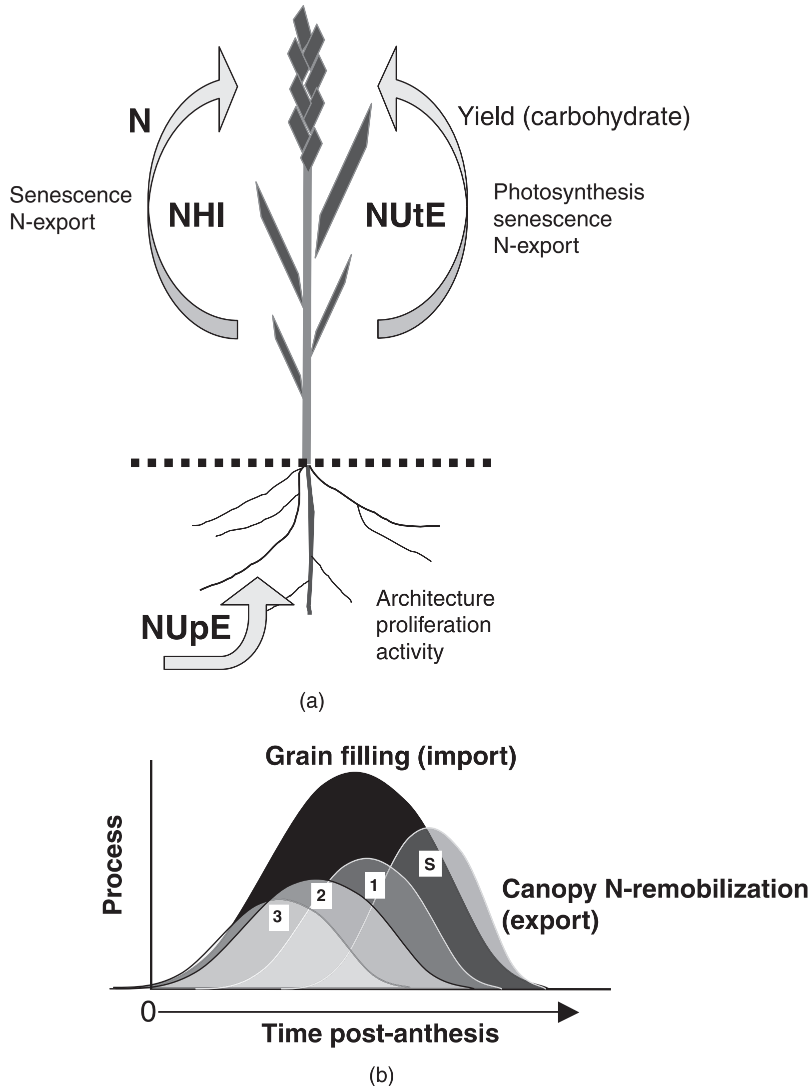

Figure 2.1 Interacting processes contributing to efficient management of nitrogen resources in a small grain cereal such as wheat. (a) NUpE depends on root parameters, and NUtE and NHI (see Table 2.1 for definitions) are dependent on canopy proliferation and consequent photosynthetic capacity and leaf longevity as well as rate of nitrogen remobilization from the canopy to grain. (b) The accumulation of nitrogen in grain depends on an export from the canopy, with a programmed sequence of senescence. Lower leaves senescence first (e.g. leaf 3 (3)) followed progressively by leaf 2, leaf 1 (flag leaf) and stem (S), ceasing production and export of photosynthate but enabling remobilization of nitrogen to the grain.

genes. Transcriptomic approaches, with their comprehensive, non-targeted coverage of the genome, therefore present a potentially important tool for the dissection of the multiple interacting processes of NUE in crops.

Sustainable crop production needs inputs matched to outputs and hence principal gains are to be achieved by minimizing losses from the system (run-off, leaching and volatilization) by optimizing application and uptake, or by improving NUtE, yield and NHI. Improving yield without increasing nitrogen inputs and without optimization of NUE will lead to a lower protein content of grain, which may be optimal for low protein applications (alcohol production, animal feeds) but compromises premium baking quality applications (e.g. bread-making). In some cases, higher protein contents are required for high nutrition-feed applications, however for rice, whilst increased nitrogen inputs will lead to higher yields, the potentially higher protein content of the grain, although improving nutritional quality will lead to a reduced eating quality, which is mainly a function of the protein-to-starch ratio. Clearly, individual crops require specific solution in relation to nitrogen nutrition.

Grain filling and hence yield in cereals such as wheat and rice are dependent on the establishment and persistence of a photosynthetically active canopy, which requires nitrogen for development. This canopy subsequently provides a major store of nitrogen which may be remobilized to the grain for protein synthesis during grain filling. Efficient use of nitrogen by a crop ideally involves a maximum remobilization from the canopy to the harvested grain; however, this will be at the expense of canopy longevity and photosynthetic capacity and therefore grain starch accumulation, as a result of the concomitant dismantling of the photosynthetic machinery. Hence, optimum yield and grain nitrogen are a compromise of the antagonistic processes of photosynthesis and nitrogen remobilization occurring during leaf senescence (Fig. 2.1). Strategies to facilitate improvements of NUE must account for these opposing processes and include modifications of soil nitrogen availability, enhancing uptake processes, or optimization of timing of canopy senescence and nitrogen remobilization. Clearly, multiple complex and interacting cellular, biochemical and developmental processes are involved which are dependent on large numbers of genes, which are also affected by agronomic practices including appropriate targeting of fertilizer application. Transcriptome profiling is an approach for dissecting these complex traits which can inform on many aspects of all these crop improvement strategies.

As NUE is directly concerned with crop production and yield, it is difficult, if not impossible, to approach this complex trait in anything other than the crop species itself or a near relative. Clearly, investigations must be performed in an appropriate environment, and whilst a case can be made for controlled environment pot-based experiments, several key traits will not be expressed under such circumstances, including the architecture of the root in relation to the soil structure and profile as well as variation in all environmental conditions to which a crop is exposed throughout its growing seasons.

2.3 Targeting NUtE processes in crop plants

Although genetic variation undoubtedly exists for both NUpE and NUtE, the latter is more tractable for analysing field-grown crops, and therefore has been the focus of recent studies. NUpE, which comprises many root-specific traits, is undoubtedly a major contributor to NUE; however, due to the difficulties of excavating and isolating field-grown roots, transcriptome analyses remain a major challenge for the future. NUtE is essentially a canopy set of traits involving both source and sink processes; in both cases complex metabolic networks have a strong developmental component as well as responding to environmental effects. Availability of microarray platforms has enabled transcriptome profiling to be applied to both sink (grain) and source (canopy) tissues, for rice, barley and wheat. By using these technologies, a large number of candidate genes involved in these processes or controlling these processes have been identified. In only a few cases, further corroborative evidence has been obtained from mutant or transgene analysis to specifically implicate roles of individually identified genes in NUE. One specific example, which was not identified by transcriptome approaches, is an NAC gene, NAMB-1, which maps to a quantitative trait locus (QTL) associated with increased grain protein, zinc and iron (Uauy *et al.*, 2006). An allele found only in ancestral wheat enhances senescence and nutrient remobilization, a role confirmed by transgenic down-regulation of expression, leading to the high nutrient contents observed, but which has a substantial detrimental effect on yield.

2.3.1 Sink processes: grain expression

Wheat grain has been subject to several transcriptome analyses; for example, a detailed analysis of the transcriptome of the developing caryopsis during wheat grain development indicates a huge number of developmentally regulated genes (14 550 out of 55 052) (Wan *et al.*, 2008). Transcriptome analysis of rice grain tissues during grain filling indicated 269 up-regulated genes (using a 21 000-gene chip) whose promoter regions have an overrepresentation of a specific AACA potential regulatory element. Furthermore, a group of transcription factors interacting with this element were identified (Zhu *et al.*, 2003).

Using a cDNA microarray for wheat (Wilson *et al.*, 2004), the influence of nitrogen nutrition on endosperm genes expressed during grain filling in wheat (Lu *et al.*, 2005) was examined. As NUE performance depends on sink tissues such as grain, it might be predicted that processes occurring in the grain are critical to NUE and that responses would be indicated by transcriptome analysis. However, whilst nitrogen supply to crops in the field clearly has a major influence on yield, this is achieved by modification of yield components sequentially (tiller number, grain number, grain size); the plasticity of the response ensures that viable grain is produced for each plant. Therefore, in this circumstance, at the cellular level, responses in specific gene expression may

be less than expected. In fact, characteristic transcriptome profiles observed in the endosperm were dependent on amount and form of nitrogen supply (Lu *et al.*, 2005); specific genes were either up-regulated or down-regulated dependent on nitrogen supply. The microarray analysis was able to accurately define treatments (low and high inorganic nitrogen inputs and comparisons with nitrogen provided in an organic form) and provided subsets of genes, diagnostic of the individual treatments. A surprising finding was that numerous nitrogen metabolism-associated genes were characteristically highly expressed in plots supplied with organic nitrogen fertilizer, probably indicating a nitrogen limitation in these endosperm tissues. This may have arisen due to the pattern of nitrogen release from the organic nitrogen fertilizer: high early season nitrogen supply and low soil nitrogen availability during grain filling. Such a pattern would result in plants with a high number of tillers and greater yield potential but a low availability at the critical developmental stage of grain filling. The identified genes overlapped with nitrogen limitation genes observed in other systems.

High grain nitrogen in wheat grain is desirable for bakers, and a potential solution is improving final partitioning to the grain, by optimizing the remobilization process from vegetative tissues to grain (NHI). High uptake of nitrogen during vegetative growth will produce a plant canopy with increased photosynthetic capacity, remaining active well into grain development. This leads to increased supply of carbohydrates as the grain expands and consequently to high yields with high carbon/nitrogen ratios, which may have a negative impact on wheat grain quality, depending on end use. A high nitrogen content is required for bread-making (>2.3% nitrogen), and a lower nitrogen content is required for feed or distilling variety grain. High NUE does not indicate the efficient transfer of nitrogen to the developing grain, but rather high grain yield, principally as carbohydrate. The high carbohydrate (starch) has the effect of diluting grain protein/nitrogen, and hence there is an inverse relationship of yield and grain nitrogen. It is extremely difficult to get high yield and high grain nitrogen without large nitrogen inputs, thus resulting in a low overall NUE.

2.3.2 Source processes: canopy senescence

As the canopy senesces, photosynthesis ceases with a negative impact on further yield increase; however, nitrogen remobilization from the canopy to the grain is enabled, as proteins are degraded and nitrogen is exported, principally as amino acids. Generally, a developmental programme of senescence is observed, with leaves senescing from the tip towards the base and with the oldest, lower leaves senescing first (Fig. 2.1(b)). This facilitates a balance of the antagonistic processes, with a continuous supply of nitrogen from recycling from senescing tissue to the grain, occurring concomitantly with the supply of carbohydrate from non-senesced tissue. This spatial and temporal variation also needs to be taken into account when sampling for transcriptome

analysis. Transcriptome analysis targeted at leaf senescence in *Arabidopsis* indicated four major patterns of temporal expression amongst 1400 significantly changing genes (out of 800 represented on the microarray) with early induction, late induction, transient induction or decreasing expression with the progress of senescence (Buchanan-Wollaston *et al.*, 2003). Functions of these genes include protein turnover, nutrient and carbon mobilization, transport, signalling and transcriptional regulation.

Continued photosynthesis and the onset of senescence processes both occur simultaneously with grain filling, and the control required to balance these antagonistic processes is a key to high yield and efficient utilization of nitrogen. Transcriptome profiling of canopy tissues during grain filling has been undertaken in rice (Gibbings *et al.*, 2003) and wheat (Gregersen & Holm, 2007; Howarth *et al.*, 2008). A transcriptome analysis of senescence in the flag leaf of wheat over a time course following ear emergence identified 140 up-regulated genes with informative annotations, including genes involved in macromolecule degradation and nutrient remobilization, as well as NAC-domain and WRKY transcription factors (Gregersen & Holm, 2007).

Processes in stems also contribute to grain filling: water-soluble carbohydrates accumulate in stems and act as a store of carbon to be used in grain filling, and accumulation may be enhanced by nitrogen limitation. A cDNA microarray representing 36 000 unique sequences has been used to compare gene expression in individual organs of wheat grown in pots at high and low nitrogen availability, with a focus on this carbon pool (Ruuska *et al.*, 2008). In stems of the nitrogen-limited plants, as compared with the high nitrogen-supplied plants, genes involved in fructan biosynthesis were up-regulated, but were then down-regulated upon sucrose feeding of individual culms. A WPK4 kinase, which was similarly regulated, was also identified and may have a role in regulation of storage of this carbon, at least in stems.

To understand processes contributing to nutrient remobilization and the interaction with nitrogen supply, the transcriptome of leaves during grain filling has been examined (Howarth *et al.*, 2008). Subsets of genes which show differential expression in a single sampled leaf (leaf 2, immediately below the flag leaf) in the weeks following anthesis, concomitant with early grain filling, can be identified, and representative subsets are shown in Figure 2.2. Subsets may be identified which show higher (Sets 2, 4, 5, 7, 9) or lower expression (Set 1) under low nitrogen supply (N1) compared to sufficient nitrogen (N2). Many of these genes show down-regulation of expression after anthesis as senescence progresses whilst some increase in abundance (Sets 2 and 9). Such analysis reveals many hundreds of nitrogen-responsive and developmentally regulated genes, many of which probably contribute to NUE. For example, genes expressed more highly at limiting nitrogen and increasing in abundance after anthesis may represent genes involved in efficient nitrogen remobilization and include known senescence-associated genes (SAGs), transporters and transcription factors. In Figure 2.2, just nine representative clusters are shown out of many hundreds of statistically significant patterns,

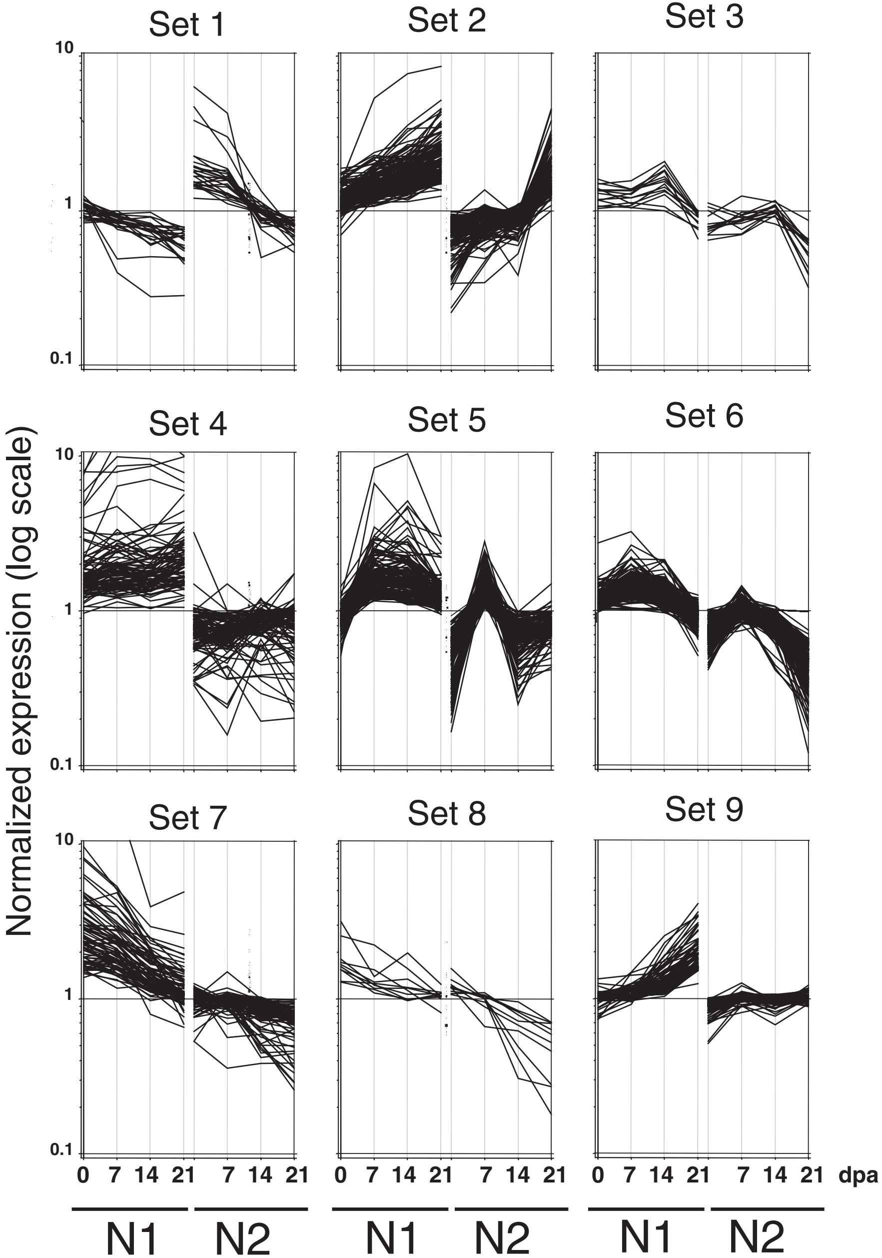

Figure 2.2 Subsets of genes developmentally regulated and influenced by nitrogen nutrition in canopy leaf tissue of wheat after anthesis. Leaf 2 was sampled from plots on the Broadbalk experiment (Rothamsted, UK) receiving 48 (N1) or 192 (N2) kg N/ha as ammonium nitrate fertilizer. Expression was determined using Affymetrix wheat microarrays. Each expression value was calculated from Affymetrix data of three biological replicates using GeneSpring microarray expression software.

with a critical limitation being a paucity of annotation and known functionality of many genes. This limitation is significant not just for wheat but also for *Arabidopsis*. Whilst undoubtedly many genes contribute to processes involved in NUE, additional analysis is required to identify the key critical genes which may have the greatest influence on NUE and which may be useful breeding or biotechnological targets.

Metabolome profiling and system analysis have been used to examine the processes contributing to nutrient remobilization from senescing canopy tissues during grain filling and to overall NUE in wheat. Combining both transcriptome data and metabolite profiles to identify co-regulated genes and metabolites, and further superimposing data on known pathways, allows greater precision and confidence in identifying genes and processes of interest. Using such an approach, nitrogen was shown to be more effectively remobilized from canopy tissue to the grain after anthesis as compared to sulphur, reflecting the greatest critical need for nitrogen to facilitate grain development (Howarth *et al.*, 2008). Analysis of amino acid pools in the grain and leaves revealed a strategy whereby amino acid biosynthesis switches to the production of glutamine during grain filling. Glutamine accumulated in the first 7 days of grain development, prior to conversion to other amino acids and protein in the subsequent 21 days. Transcriptome analysis indicated that a down-regulation of the terminal steps in many amino acid biosynthetic pathways occurs to control pools of amino acids during leaf senescence. Furthermore, nuclear magnetic resonance (NMR) based-metabolite profiling revealed significant effects on the metabolite profile of leaves, but not grain under suboptimal nitrogen or sulphur supply. This indicated that under nutrient-stressed conditions, source tissue metabolism adapts to maximize nutrient use efficiency, whereas sink tissues appear to be buffered from nutritional status and are developmentally controlled. Whilst there were clear impacts on total yield, the cellular metabolic processes occurring in individual grains did not appear to be affected by nutrient availability. These data contrast with the study of Lu *et al.* (2005), where an impact on the grain transcriptome was evident in response to nitrogen supply, illustrating a difficulty of the variability of field-grown materials. However, there is a certainty that major targets for increased yield and NUE are likely to be in the canopy and may be associated with post-anthesis canopy longevity of function as well as in nitrogen recycling within the plant. The combination of transcriptome profiling and system-based studies will highlight the key processes and provide targets for manipulation, including genes which were not previously associated with these processes.

2.4 Validating candidate genes by correlating gene expression with complex traits

An approach to the resolution of transcriptomic data and the identification of key genes is to exploit genetic variation in expression of key traits. An

example of such an approach was a comparison of near-isogenic barley lines with contrasting grain protein content and associated accelerated leaf senescence (Jukanti *et al.*, 2008). Both flag leaf and kernel transcriptomes using an Affymetrix array (22 000 probe sets) were analysed at two time points after anthesis (14 and 21 dpa). The analysis indicated an up-regulation of both plastidal and extraplastidal proteases in the germplasm with accelerated leaf senescence, and also identified further novel candidate genes involved in senescence processes including leucine-rich repeat transmembrane kinases and a glycine-rich RNA-binding protein. Potentially, an even more powerful approach was the application of transcriptome profiling to a doubled haploid population (41 lines and two field locations for experimentation) which led to the identification of expression polymorphisms in developing seeds which could be mapped to produce so-called eQTLs (Jordan *et al.*, 2007). A gene mapped as an eQTL is not necessarily physically located in its mapped position, rather in some cases, control of its expression is located at that locus, and hence this approach allows the mapping of specific *cis-* and *trans-*acting regulatory elements within the genome. Such approaches would be very powerful if applied to a mapping population screened at different nitrogen inputs.

Variation in NUE and the component traits, NUpE and NUtE, occurs in wheat (Ortiz-Monasterio *et al.*, 1997), and whilst an important goal is the identification of genes responsible for this variation, the variation itself may be exploited as a means for key gene identification; therefore, utilizing this variation in combination with transcriptome profiling is a useful approach. To specifically investigate variation in NUtE and identify underpinning contributing genes, a range of European wheat varieties with contrasting phenotypes were subjected to extensive physiological and agronomic studies, combined with transcriptome profiling approaches. The five varieties (Maris Wigeon, an older tall variety; Hereward, a high-protein content, breadmaking variety; Riband, a low-protein variety; Soissons, an early-flowering variety; Istabraq, a late-flowering variety) were grown in a replicated field experiment at multiple levels of nitrogen, and measurements were made of agronomic and physiological performance. Variations in NUtE for the wheat varieties at three rates of nitrogen input are shown in Figure 2.3. In all varieties, NUtE decreases with increasing nitrogen as yield does not increase proportionally to applied nitrogen. NUtE also differs between varieties with the feed/biscuit (high starch) varieties having greater NUtE than the high protein, bread-making types due to their higher yields. Finally, the response curves for NUtE are not always parallel with Riband, showing a much greater decrease in NUtE with increasing nitrogen compared to Istabraq in the field trial shown. These differences in this complex agronomic trait must be explained by genetic determinants of protein-versus-starch synthesis, both as a consequence of control of expression of protein in the grain but also due to factors controlling photosynthetic activity in the canopy, rates of canopy senescence and difference in nitrogen remobilization.

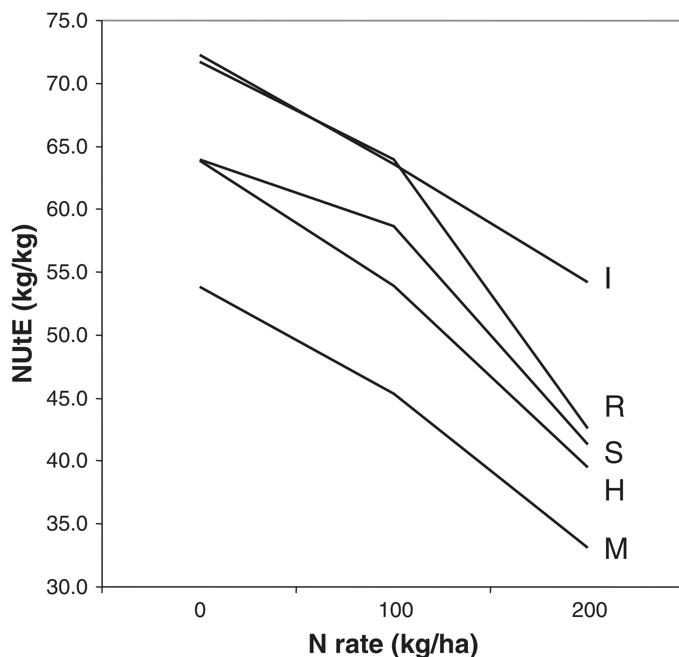

Figure 2.3 The effect of nitrogen input and genotype interactions on NUtE for five varieties as determined in a field trial at three rates of nitrogen supply (at Rothamsted, UK, with triplicate plots in 2006; P.B. Barraclough, J.R. Howarth and M.J. Hawkesford, unpublished). NUtE is kg of grain (100% dry matter) per kg of applied nitrogen fertilizer. Varieties were Istabraq (I), Riband (R), Soissons (S), Hereward (H) and Maris Widgeon (M).

This field trial was replicated with the addition of a short-stature variety, Welford, and in addition to physiological measurements, leaf samples were collected for transcriptome profiling (J.R. Howarth & M.J. Hawkesford, unpublished). Transcriptome analysis was performed on leaf 2 samples taken at 7 and 21 days after anthesis, and Figure 2.4 shows a hierarchical clustering of the replicates and the treatments based on genes which had significant changes in expression as a function of the nitrogen treatment. In each case, biological samples were taken from three separate field plots, and in all cases, the transcriptome profiles of the replicates clustered together. Of the 62 309 unique probe sets on the Affymetrix array, 22 211 were responsive in at least one replicate set by greater than 1.5-fold expression change between means of replicates and 952 probe sets were specifically and consistently responsive to the nitrogen treatment. The two nitrogen treatments clustered completely independently, as would be expected, since this was the basis of the gene list selection, but also highlighting the major differences in metabolism in the canopy tissue, occurring as a result of nitrogen supply. Within these major two clusters, the two time points also clustered independently, reflecting the developmental changes occurring in the leaf over this period and which were

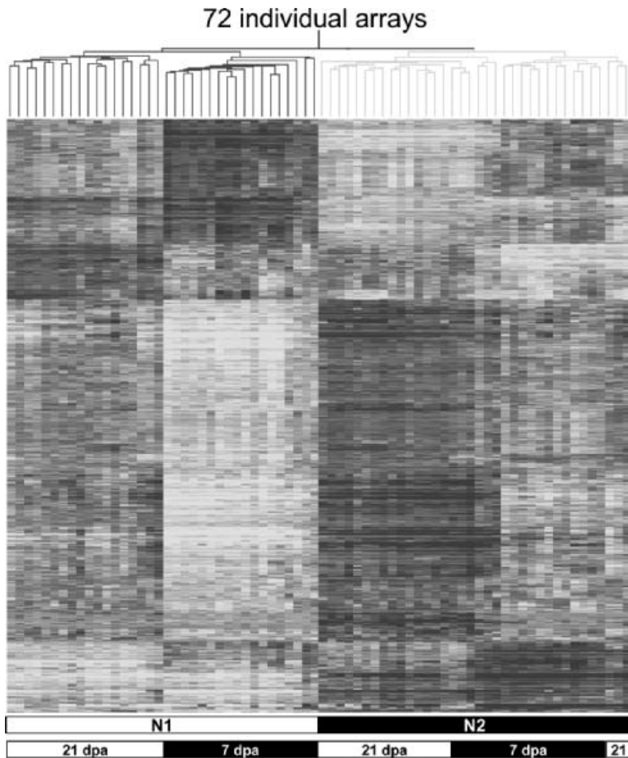

Figure 2.4 Hierarchical clustering by expression profile (GeneSpring condition/gene tree) of the 952 most nitrogen-responsive genes from six wheat varieties at 50 (N1) and 200 (N2) kg nitrogen/ha between 7 and 21 dpa (standard correlation), grown in the field (in 2006) with three biological replications. The key identifies the nitrogen level and days after anthesis (dpa). Varieties are as listed in the legend to Figure 2.3, with the addition of Welford, a very short-stature variety.

occurring in both nitrogen treatments. There was one exception to this pattern with the 21 dpa (days post-anthesis) samples from one variety, Soissons, clustering with the 7 dpa data. This variety exhibited slow maturation and delayed leaf senescence, hence, the similarities of the 7 and 21 dpa transcriptomes. As the 952 nitrogen-responsive probe sets represent a similar number of genes, there is clearly a need for further refinement to determine the most relevant genes amongst this group. To address this problem and further resolve the identified nitrogen-responsive gene lists, genes were identified whose expression profile was highly positively or inversely correlated with the measured agronomic and physiological traits including yield, grain and leaf nitrogen content, nitrogen-export ability (the change in nitrogen content of the leaf within the period measured) as well as NUE and its components. Figure 2.5 shows the degree of correlation of 31 highly correlating genes with four selected parameters: export of nitrogen from leaf 2 between 7 and 21 days after anthesis, overall crop NUtE, NHI and crop total nitrogen uptake. The

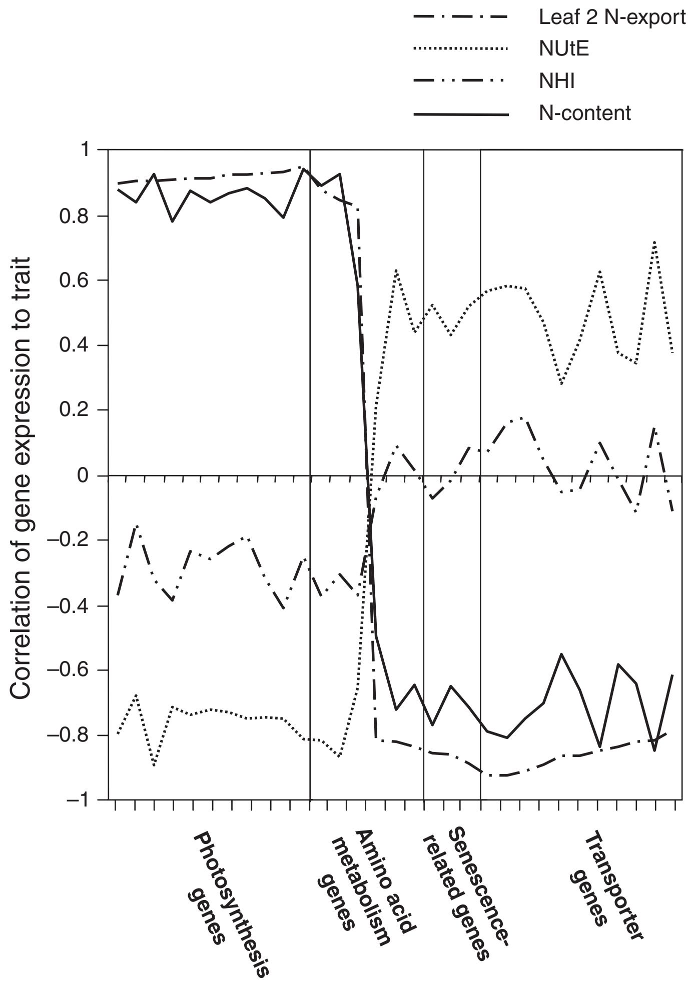

Figure 2.5 Correlation of expression of individual genes with specific nitrogen-related traits. Correlations for 31 genes (divided into four functional groups) to four traits are indicated. The selected genes were those that were both highly expressed and most strongly correlated (positive or inverse standard correlation), with the physiological and agronomic NUE parameters are shown across six wheat varieties grown in the field at two nitrogen input levels (50 and 200 kg nitrogen/ha) and sampled at 7 and 21 dpa. Varieties are as indicated in the legends to Figures 2.3 and 2.4.

31 genes are grouped into functional groups and either positive or inverse correlation is indicated.

Figure 2.5 indicates that expression of the set of photosynthesis-related genes and some genes involved in amino acid metabolism correlate positively with leaf nitrogen content and potential to export nitrogen from the leaf, and inversely correlate with NUtE, reflecting the positive effect nitrogen has on canopy photosynthesis; the low NUtE correlation is a consequence of these

same tissues being from the treatments with high nitrogen inputs; the low correlation with expression of transporters reflects the fact that maximum export is occurring, and presumably these transporters are involved, when nitrogen is low, either at 21 dpa or under low-nitrogen fertilizer conditions. Conversely, the expression of the genes encoding the transporters and genes involved in senescence processes (principally proteases) and some genes involved in amino acid biosynthetic pathways including one glutamine synthetase isoform (nitrogen metabolism group) positively correlates with treatments with high NUtE, where yield per unit of available nitrogen is high, suggesting that high expressions of these are genes associated with efficient use of nitrogen. In all cases, with either high positive or high inverse correlation, the inference is that these genes are related to traits contributing to NUE. NHI generally shows a poor correlation with all of these genes and thus is clearly an independent trait, probably influenced more strongly by stature. Focusing on genes whose expression shows high correlations to relevant physiological and agronomic characteristics, both across varied germplasm and with contrasting developmental and nutritional status, provides a candidate list of genes vital to NUE processes worthy of further analysis. However, the number of contributing genes still remains high, which is to be expected for such a complex set of interacting traits. A goal remains to find global regulators that coordinate the genes and pathways, for example the transcription factors.

A large number of transcription factors correlated with agronomic factors such as leaf nitrogen content and nitrogen export in this study of wheat germplasm with widely varying NUE characteristics. Expression patterns of four selected transcription factors are shown in Figure 2.6. These were selected for their high level of positive or inverse correlation to leaf nitrogen export in all six varieties, whether grown at high- and low-nitrogen fertilizer application.

Importantly, expression of these transcription factors in the canopy tissues after anthesis correlates with yield, NUE parameters as well as activity of nitrogen remobilization following anthesis (Figs 2.1 and 2.2). Each of the transcription factors belongs to a large gene family, and the functions of most members of these families are not known. Manipulation of expression of these transcription factors may be expected to have profound effects on nitrogen remobilization, yield and NUE parameters. The transcription factors are likely to 'globally' control expression of large numbers of genes, either directly or in cascades, including the large subsets identified in our current studies which include nitrogen metabolism, proteolytic enzymes, transporters and photosynthesis-related genes. The genes have significant potential as targets for marker-assisted breeding (MAB) of wheat with improved NUE.

2.5 Prospects

The key to the genetic improvement of germplasm for improved NUE, and hence reduced nitrogen inputs, as well as improving yield, is the resolution of

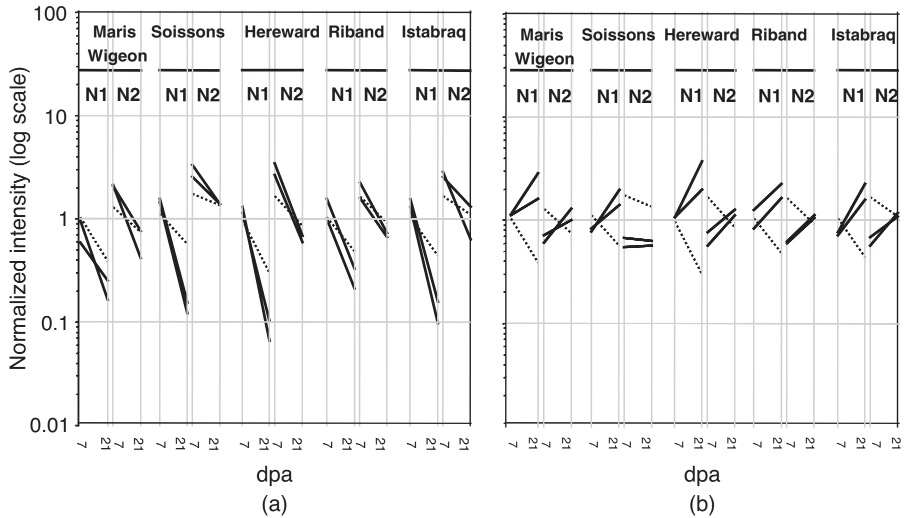

Figure 2.6 Graphical representation of expression (solid lines) of four selected transcription factor genes in leaf 2 of field-grown wheat plants at 50 and 200 kg nitrogen/ha fertilizer application, between 7 and 21 dpa and corresponding to nitrogen-remobilization data (leaf 2% N, broken lines). (a) Nitrogen positively correlated genes. (b) Nitrogen inversely correlated transcription factors. The five varieties shown are presented in order of increasing NUtE. Varieties were Istabraq (I), Riband (R), Soissons (S), Hereward (H) and Maris Widgeon (M), from left to right.

the target traits and subsequent identification of the respective genes and loci underpinning the trait variation. Variation in germplasm needs to be linked to the involvement of specific genes, which will be the targets for germplasm improvement; these genes may be components of biochemical or developmental pathways or be regulatory elements such as transcription factors involved in critical signal and regulatory cascades. The key target traits fall into groups responsible for uptake efficiency or utilization efficiency, including portioning of nitrogen in the final crop (NHI). The numbers of candidate genes which may be the keys to crop improvement remain high and require further studies targeted at validation, for example with mutants or transgenic materials. Other approaches to validate candidate genes involved in NUE will include additional analysis of diverse germplasms combined with varied supply of nitrogen. As already outlined, it is difficult to replicate field conditions in the laboratory and therefore field-grown material is required, and future studies will need to address the issues of analysis of the roots and contributions to NUpE. Transcriptome profiling will continue to have an important part to play in this process. All the analyses indicated in this chapter contribute to identifying components of the molecular networks underpinning yield, NUE and nitrogen allocation in crops; however, a critical understanding of these networks will only be achieved with a more complete genome annotation

and profiling of critical systems, exploiting the variation that is the target of much of the research.

Acknowledgements

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Chapter 3

ENERGETICS OF NITROGEN ACQUISITION

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Abstract: Plants employ a variety of mechanisms to acquire nitrogen from their environment ranging from carnivory to symbiotic relationships with bacteria. Most plants, however, obtain the majority of their nitrogen as nitrate and ammonium that their roots absorb from soils. To compete successfully against soil microorganisms for soil nitrate or ammonium, plants adjust their growth, development and physiology to exploit the distinct properties of each ion.

Nitrate is more mobile than ammonium through most soils. Plants can store high concentrations of nitrate, but not ammonium, within their tissues. Yet, assimilation of nitrate into amino acids is far more energy-intensive than that of ammonium. Plants offset some of the energy costs of nitrate assimilation by coupling it with photorespiration. Rising carbon dioxide levels in the atmosphere inhibits photorespiration and, thereby, nitrate assimilation. Therefore, ammonium and nitrate management will become even more critical in the future.

Keywords: acid growth; ammonium; ammonium toxicity; nitrate; nitrate assimilation; photorespiration

Nitrogen is a constituent of many organic compounds in plants including all amino acids and nucleic acids. As such, plants require a greater amount of nitrogen than any other mineral elements, and its availability generally limits the productivity of natural and agricultural ecosystems (Epstein & Bloom, 2005). To boost crop yields, farmers apply over 100 million metric tons of nitrogen fertilizer per year worldwide (FAOSTAT, 2008). This nitrogen fertilizer – if one includes its manufacture, distribution and application and its microbial transformations in soils – is responsible for more than half of the total energy consumed in agriculture (Brown *et al.*, 1987) and the majority of greenhouse gas emissions from agriculture (Bloom, 2010). A large portion

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of the nitrogen fertilizer applied to crops does not become plant material; rather it leaches into groundwater or volatizes into the atmosphere. This not only wastes a valuable resource, but also endangers human health (National Research Council, 1989). Clearly, improving the efficiency with which plants acquire nitrogen from the environment and incorporate it into their organic matter is of critical importance.

Nitrogen as a chemical element has extraordinary versatility in oxidation–reduction reactions, an attribute that makes it central to biogeochemical cycles and plant metabolism. At one end of the spectrum is the highly oxidized compound nitrate (NO_3^-) in which nitrogen has a valence of +5. In the middle of the spectrum is dinitrogen gas (N_2) in which the element has a valence of zero. The reduced compounds at the opposite end of the spectrum are ammonium (NH_4^+) and organic compounds in which nitrogen has a valence of −3.

Conversions among the various nitrogen forms are the most energy-intensive reactions in life. Plants are generally between 1 and 2% nitrogen on a percentage dry weight basis, yet expend somewhere about 25% of their energy on nitrogen acquisition (Bloom *et al.*, 1992; Cousins & Bloom, 2004). For some perspective on the enormous energies involved, these processes expend the energy equivalent of between 12 and 18 adenosine triphosphates (ATPs) per inorganic nitrogen assimilated into amino acids, whereas most biochemical reactions expend the energy equivalent of 1 or perhaps 2 ATPs. It is even more impressive that when these nitrogen reactions run in reverse – say, from NH_4NO_3 to N_2 – they cause an explosion. Nearly all conventional explosives (e.g. TNT, nitroglycerin and dynamite) depend on the rapid oxidation of nitrogen.

This chapter first presents the energetics of scavenging various forms of nitrogen from the environment and assimilating these various forms into organic compounds within plants. Next, the chapter describes the influence of these processes on plant growth and development. Finally, the chapter speculates on the relationship between nitrogen energetics and the past, present and future distribution of plant species.

3.1 Availability of nitrogen in the environment

The vast majority of nitrogen on earth is in the form of dinitrogen gas (N_2) that makes up 78% of the atmosphere. Most organisms cannot directly access this huge nitrogen reservoir because the exceptionally stable triple covalent bond between the two nitrogen atoms ($N{\equiv}N$) renders this form an inert or 'noble' gas. Breaking this triple bond to generate compounds such as ammonia (NH_3) or nitrate (NO_3^-), which are accessible to most organisms, requires an enormous input of energy. Nonetheless, chemical reactions of such proportions occur during the industrial or natural processes known as nitrogen fixation.

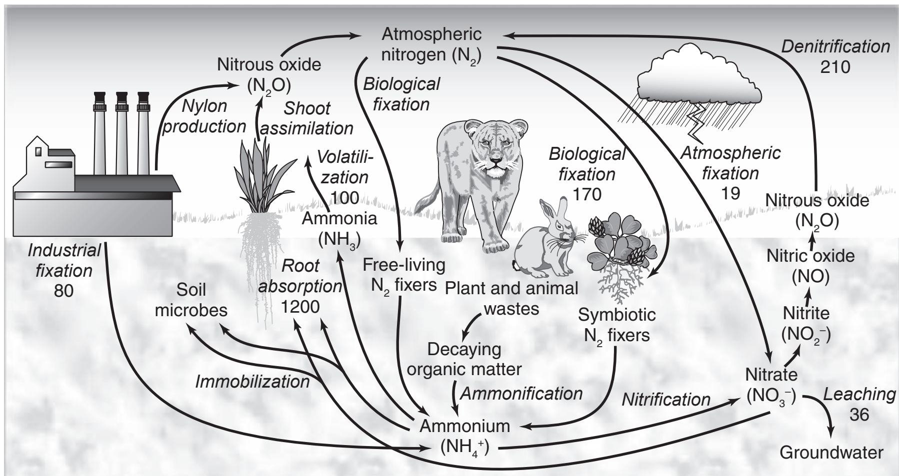


Figure 3.1 Terrestrial nitrogen cycle. Fluxes are in Tg per year (Galloway *et al.*, 2004; Epstein & Bloom, 2005)

Industrial nitrogen fixation, which produces agricultural fertilizer, exceeds 100×10^{12} grams of nitrogen per year (Fig. 3.1). Natural nitrogen fixation proceeds at a rate of about 232×10^{12} grams of nitrogen per year. About 2% of this naturally fixed nitrogen is the product of lightning, whereby the electrical discharge converts water vapour and oxygen into highly reactive hydroxyl free radicals, free hydrogen atoms and free oxygen atoms that attack dinitrogen gas to form nitric acid (HNO_3). An additional 1% of the naturally fixed nitrogen derives from photochemical reactions between gaseous nitric oxide and ozone to produce nitric acid. The remaining 97% results from biological nitrogen fixation in which bacteria or blue-green algae (cyanobacteria) fix molecular nitrogen into ammonium (NH_4^+).

Nitrogen, once fixed into NH_4^+ or NO_3^-, enters a biogeochemical cycle and passes through several organic or inorganic forms before it eventually returns to molecular nitrogen (Fig. 3.1). Amino acids, NH_4^+ and NO_3^-, which are generated through fixation or released through decomposition of soil organic matter, become the object of intense competition among plants and micro-organisms. To remain competitive, plants have developed diverse mechanisms for scavenging these nitrogen forms as quickly as possible. These mechanisms range from routine to bizarre – from standard active transport of soil nutrients to carnivory of small animals or symbiotic relationships with micro-organisms. To what degree do plants rely on different mechanisms for nitrogen scavenging? The following focuses mainly on NH_4^+ and NO_3^- acquisition, but provides cursory treatments of carnivory, nitrogen fixation, mycorrhizal associations and organic nitrogen scavenging.

3.2 Curiosities

Over 600 species, scattered throughout the plant kingdom and native to nutrient-poor sites, practise carnivory (Ellison & Gotelli, 2001). These species attract and kill small animals that have the misfortune to cross their path, digest them, and thus obtain their nitrogen and phosphorus (Darwin, 1875; Adamec, 1997; Ellison *et al.*, 2003). For example, sundews (*Drosera* sp.) when fed insects labelled with the heavy isotope of nitrogen ^{15}N, developed a ratio of $^{15}N/^{14}N$ in their tissues, indicating that the plants obtained between 15 and 87% of their nitrogen through carnivory (Dixon *et al.*, 1980; Schulze & Schulze, 1990; Schulze *et al.*, 1991). Carnivorous plants, although intriguing, expend more energy in acquiring nutrients than non-carnivorous plants growing in similar habitats (Ellison, 2006); therefore, carnivory remains relatively rare.

Far more central to agriculture and forestry are plants that form intimate relationships with nitrogen-fixing prokaryotes. Common are associations between higher plants and free-living nitrogen-fixing bacteria in soils, where root exudates and plant detritus provide an energy source for the bacteria, while turnover of the bacteria enhances soil nitrogen and its availability to the plants (Vessey, 2003). In another type of relationship, nitrogen-fixing bacteria grow in non-specialized intercellular spaces of grasses such as sugarcane and *Miscanthus*, where plants exchange carbohydrate for reduced nitrogen from the bacteria (Reis *et al.*, 2000). A third type of relationship is a mutualistic symbiosis in which plants house nitrogen-fixing bacteria within specialized plant organs. Such symbioses occur between rhizobial bacteria and legumes (about 650 genera of one family), *Frankia* bacteria and actinorhizal plants (25 genera, in 8 different families, in 3 different orders), rhizobial bacteria and *Parasponia* (5 species in 1 genus), and cyanobacteria and cycads (11 genera in 3 families) (Vessey *et al.*, 2005).

Relatively few plant species conduct symbiotic nitrogen fixation because it is almost prohibitively expensive in terms of energy. In theory, nitrogen fixation expends the equivalent of about 16 ATPs per N_2 converted to NH_4^+, or about 36% more energy than NO_3^- reduction to NH_4^+ (Pate & Layzell, 1990). In practice, the differences in energy expenditures between N_2 and NO_3^- assimilation are slightly more (Bloom *et al.*, 1992; Voisin *et al.*, 2003; Lundquist, 2005). Little wonder, therefore, that nitrogen-fixing plants shut down this process whenever soil NH_4^+ or NO_3^- becomes available (Salvagiotti *et al.*, 2008).

Most higher plants form symbiotic relationships with mycorrhizae. Mycorrhizae increase the physiologically absorbing surface area of a root system and enhance the decomposition of soil organic nitrogen through the excretion of ectoenzymes (He *et al.*, 2003), but the extent to which these fungi boost root scavenging of soil nitrogen is still an open question. Although mycorrhizae transfer large amounts of nitrogen from the soil to their host plants in several forms including NH_4^+ (He *et al.*, 2003; Govindarajulu *et al.*, 2005), mycorrhizal sequestration of available soil nitrogen may limit the nitrogen supply to the

host. Consequently, mycorrhizal plants often have a lower nitrogen status than non-mycorrhizal ones (Hawkins *et al.*, 2000; Hobbie *et al.*, 2008).

Higher plants can absorb exogenous amino acids (Lipson & Nasholm, 2001) and proteins (Paungfoo-Lonhienne *et al.*, 2008) without microbial assistance. In some locations at certain times, such as springtime in arctic and alpine ecosystems, substantial amount of nitrogen becomes available in these organic forms, and plants accumulate quantities of nitrogen that seem to exceed those available in inorganic forms (Neff *et al.*, 2003). Generally, however, higher plants obtain only a minor fraction of their nitrogen from the soil in organic forms because they cannot compete with micro-organisms for these high-energy compounds (Lipson & Nasholm, 2001).

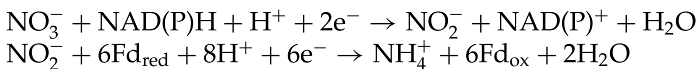
3.3 Mineral nitrogen

A vast majority of plants obtain nitrogen directly from the environment via root absorption of soil NO_3^- and NH_4^+ (Williams & Miller, 2001). Plant performance – its fitness, yield, nutrient efficiency or susceptibility to biological or environmental stress – generally hinges on the ability of the plant to obtain this mineral nitrogen. Even carnivorous, nitrogen-fixing or mycorrhizal plants curtail these activities whenever NO_3^- or NH_4^+ is available from the soil.

3.3.1 Nitrate

Microbes in well-aerated, temperate soils rapidly convert organic nitrogen and NH_4^+ to NO_3^-. Therefore, the availability of NO_3^- can sometimes exceed that of amino acids or NH_4^+ by over an order of magnitude. This NO_3^- moves relatively freely through the soil (Nye & Tinker, 1977) because NO_3^-, as an anion, does not bind to the cation exchange complex found in most soils and because all NO_3^- salts are highly soluble. Consequently, NO_3^- might seem to be the preferred source of nitrogen because of its availability and mobility.

Unfortunately, NO_3^- is the most oxidized form of nitrogen, and its assimilation requires a reduction first to nitrite (NO_2^-) catalysed by nitrate reductase and then to NH_4^+ catalysed by nitrite reductase (Fig. 3.2):



$$NO_3^- + NAD(P)H + H^+ + 2e^- \rightarrow NO_2^- + NAD(P)^+ + H_2O$$
$$NO_2^- + 6Fd_{red} + 8H^+ + 6e^- \rightarrow NH_4^+ + 6Fd_{ox} + 2H_2O$$

These reactions in theory and in practice expend the equivalent of 10 ATPs (Bloom *et al.*, 1992). Thus, although most soil organisms have the ability to absorb and assimilate NO_3^-, they avoid doing so until they deplete the medium of NH_4^+ or amino acids. For example, some soil bacteria derive 40% of their nitrogen from NO_3^- (Keeney *et al.*, 1971), but their growth rate under NO_3^- nutrition is one-third of that under NH_4^+ or amino acid nutrition (Wray *et al.*, 1996). In most micro-organisms, the presence of NH_4^+ strongly inhibits

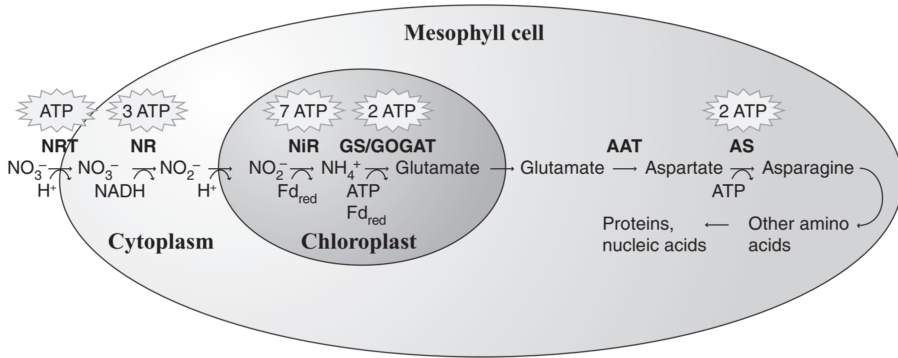


Figure 3.2 Nitrate (NO_3^-) acquisition in leaves. Symporters (NRT) bring NO_3^- and a proton into a mesophyll cell. Nitrate reductase (NR) converts NO_3^- into nitrite (NO_2^-). The NO_2^- is translocated as HNO_2 into the stroma of a chloroplast, where nitrite reductase (NiR) uses the energy of six reduced ferredoxins (Fd_r) to convert it into ammonium (NH_4^+). The sequential action glutamine synthetase (GS) and glutamate synthase (GOGAT) convert NH_4^+ into glutamate. Glutamate becomes converted into other amino acids in the cytoplasm (Epstein & Bloom, 2005).

absorption and assimilation of NO_3^- (Moreno-Vivian *et al.*, 1999; Lundberg *et al.*, 2004). Indeed, the fact that most soil microbes use NO_3^- as a nitrogen source only under duress may be responsible for the prevalence of NO_3^- in many soils.

By contrast, plants in temperate zones, particular those in open canopies, tend to rely on NO_3^- for a major portion of their nitrogen requirements (Haynes, 1986). Under such circumstances, CO_2, water or nutrients may limit plant productivity more than sunlight, and so the energy requirements of NO_3^- assimilation may prove less burdensome. This does not mean that these plants depend on NO_3^- as their sole nitrogen source in temperate ecosystems; rather NO_3^- is often a major nitrogen source.

Roots contain several types of transport systems for NO_3^-, some of which operate in the micromolar range and others in the millimolar range (Lea & Azevedo, 2006). Plants, however, absorb most of their NO_3^- during flushes in which groundwater concentrations of NO_3^- reach 1 mM (Nolan & Hitt, 2006). Cytoplasmic NO_3^- concentrations in higher plants are between 2 and 6 mM (Ritchie, 2006; Miller & Smith, 2008). Because of this relatively small concentration gradient between the rhizosphere and roots, active transport of NO_3^- in theory (Glass, 1988) and in practice (Bloom *et al.*, 1992) usually expends only the equivalent of 1 ATP.

Both roots and shoots in most plants have the capability to assimilate NO_3^-. The relative extent to which NO_3^- reduction takes place in one organ or the other, however, depends on a number of factors, including the level of NO_3^- supplied to the roots and plant species. Generally, when the roots receive small amounts of NO_3^-, its reduction takes place primarily in the roots. It seems reasonable that when nitrogen availability is limited, more of it would

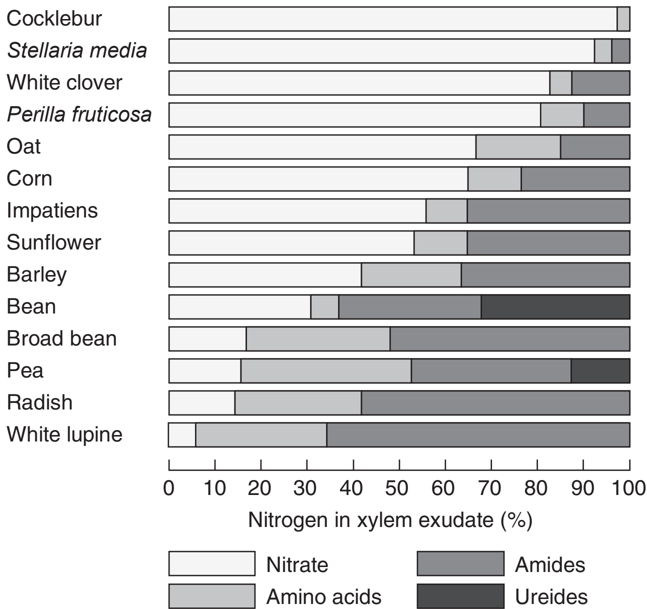


Figure 3.3 Relative amounts of nitrate and other nitrogen compounds in the xylem exudate of various plant species. The plants were grown with their roots exposed to nitrate solutions, and xylem sap was collected by severing of the stem. Note the presence of ureides in bean and pea, which export ureides from their nodules. After Pate (1973).

remain in the roots and promote root growth (Brouwer, 1967). As the supply of NO_3^- increases, roots translocate a greater proportion of NO_3^- that they absorb to the shoot, where it is assimilated and promotes shoot growth (Marschner, 1995).

The balance between root and shoot NO_3^- metabolism – assessed from the proportion of nitrate reductase activity in each of the two tissues or from the relative concentrations of NO_3^- and reduced nitrogen in the xylem sap – also varies from species to species (Fig. 3.3). In plants such as the cockleburr (*Xanthium* sp.), NO_3^- metabolism is restricted to the shoot, whereas in others such as white lupine (*Lupinus* sp.), the root assimilates most of the NO_3^- absorbed from the soil. Generally, species native to temperate regions rely more heavily on root NO_3^- assimilation than species with tropical or subtropical origins (Andrews, 1986).

We found that NO_3^- assimilation depends on photorespiration in the shoots of C_3 plants (Bloom *et al.*, 2002b; Searles & Bloom, 2003; Rachmilevitch *et al.*, 2004), but not in C_4 plants (Cousins & Bloom, 2003, 2004). Exposure to elevated CO_2 or low O_2 inhibited NO_3^- assimilation in C_3 plants (Fig. 3.4) because it:

- Impeded NO_2^- translocation into the chloroplast (Bloom *et al.*, 2002b)
- Increased competition between carbon fixation and nitrite reduction for reduced ferredoxin in the chloroplast (Robinson & Baysdorfer, 1985)

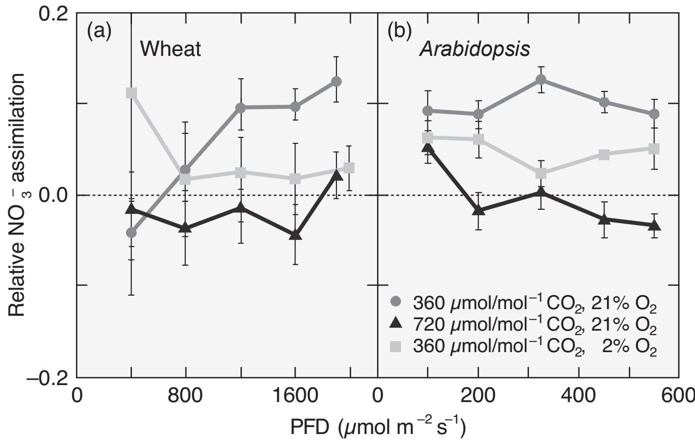


Figure 3.4 Relative NO_3^- assimilation as a function of light intensity (PFD). NO_3^- assimilation was monitored from the change in assimilatory quotient (CO_2 consumption/O_2 evolution) when the nitrogen source was shifted from nitrate to ammonium (ΔAQ). Shown are mean ± SE for (a) wheat (*Triticum sativum*) and (b) *Arabidopsis thaliana*. NO_3^- photoassimilation became significant only under the condition of 360 μmol/mol CO_2 and 21% O_2, and sometimes high light levels (Rachmilevitch *et al.*, 2004).

- Decreased photorespiration and thus the flux of malate through cytoplasm and NADH available for nitrate reduction (Igamberdiev *et al.*, 2001)

Accordingly, CO_2 enhancement of shoot growth was cut in half when wheat plants received NO_3^- rather than NH_4^+ (Fig. 3.5). In C_4 plants, the first carboxylation via PEP carboxylase generates sufficient malate in the cytoplasm of mesophyll cells to support NO_3^- assimilation independent of CO_2 or O_2 concentrations.

3.3.2 Ammonium

Most soil particles have fixed negative charges on their surfaces, and thus most soils have a substantial cation exchange capacity. NH_4^+ adsorbs onto these exchange sites with a tenacity stronger than all but protons, calcium or magnesium ions. Consequently, the availability of NH_4^+ from soils generally shows much less variation, both seasonally and spatially, than the availability of other nitrogen forms, particularly NO_3^- (Jackson & Bloom, 1990; Bloom, 1997b). Accordingly, roots have several constitutive transport systems that have affinities for NH_4^+ in the micromolar range (Loque & von Wiren, 2004).

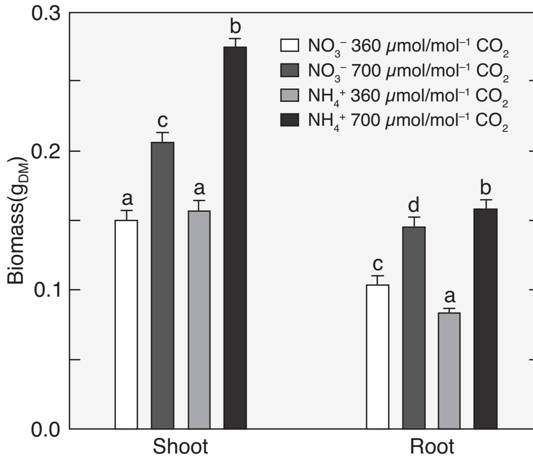
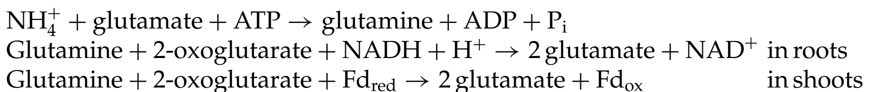


Figure 3.5 Biomass (g dry mass) per plant of wheat seedlings grown for 14 days in controlled environment chambers at 360 or 700 μmol/mol CO_2 and under NO_3^- or NH_4^+ nutrition. Shown are mean ± SE for four replicate experiments, each with eight to ten plants per treatment. Treatments labelled with different letters differ significantly ($P <$ 0.05) (Bloom *et al.*, 2002b).

Ammonium, if it accumulates to high levels within living tissues, dissipates proton gradients across membranes and thereby disrupts organelle electron transport and eventually causes cell death. To avoid such toxicity, plant cells rapidly convert NH_4^+, which is absorbed from the rhizosphere or generated from NO_3^- assimilation or photorespiration, into amino acids. The primary pathway for this conversion in higher plants (Fig. 3.2) involves the sequential actions of glutamine synthetase (GS) and glutamate synthase, also known as glutamine:2-oxoglutarate aminotransferase (GOGAT) (Lea *et al.*, 1992):



$$NH_4^+ + \text{glutamate} + \text{ATP} \rightarrow \text{glutamine} + \text{ADP} + P_i$$
$$\text{Glutamine} + \text{2-oxoglutarate} + \text{NADH} + H^+ \rightarrow 2\,\text{glutamate} + \text{NAD}^+ \quad \text{in roots}$$
$$\text{Glutamine} + \text{2-oxoglutarate} + \text{Fd}_{red} \rightarrow 2\,\text{glutamate} + \text{Fd}_{ox} \quad \text{in shoots}$$

NH_4^+ assimilation in roots or shoots, therefore, expends the equivalent of 2 ATPs.

In summary, the disadvantages of NH_4^+ as a nitrogen source are its potential for toxicity and its binding to the soil cation exchange capacity. Its advantages are that it may be the major nitrogen form available from the soil at certain times and places and that its assimilation expends less energy than NO_3^- reduction or N_2 fixation. Many plants prefer NH_4^+ as a nitrogen source, particularly those native to environments where flooding leaches NO_3^- from the soil or where soil acidity inhibits nitrification (Bloom, 1997b). This does not mean that these plants depend on NH_4^+ as their sole nitrogen source; rather NH_4^+ is often their major nitrogen source.

3.4 Plant growth and development

The rhizosphere from which plants must extract nitrogen is highly heterogeneous, both spatially and temporally. For example, NO_3^- concentrations in a soil may range a 1000-fold over a distance of centimetres or over the course of hours (Bloom, 1997b). Plant roots themselves modify their surroundings: they deplete nutrients, alter rhizosphere pH through ion exchange (Smart & Bloom, 1998; Taylor & Bloom, 1998), and support soil microbes through exudates or cell death. Given such heterogeneity, plant roots must be in the right place at the right time and with the right equipment (Bloom, 1994). Plants depend on various tropisms (e.g. gravitropism, thigmotropism, chemotropism and hydrotropism) to guide root growth towards soil resources (Bloom *et al.*, 2004). In particular, NO_3^- and NH_4^+ themselves may be responsible for the root developmental responses (Forde, 2002).

We conducted an experiment to clarify whether NO_3^- and NH_4^+ themselves serve as signals for determining root proliferation (Bloom *et al.*, 2002a). Maize seedlings (*Zea mays* cv. Dekalb), whose roots were exposed to 1 mM $CaSO_4$ for 2 days, were then exposed to nutrient solution containing 1 mM $CaSO_4$ and either 5.0 mM KCl, 5.0 mM KNO_3, 0.1 mM KCl or 0.1 mM NH_4Cl for another 2 days. The NH_4^+ concentrations were lower than those of NO_3^- to avoid ammonium toxicity. Subsequently, all the treatments received the same nutrient solution (one-tenth strength of a modified Hoagland solution; Bloom, 2002) for additional 6 days. We measured the roots three times: first, before the different treatments were applied; second, when the different treatments ceased; and finally, 6 days after the treatments. These measurements permitted us to identify which parts of the seminal root initiated before, during or after the treatments.

There were several differences among the treatments (Fig. 3.6). In the root zone initiated during the NO_3^- treatment, lateral roots were longer (Fig. 3.6b). This response appeared to require direct exposure of young laterals to external NO_3^- because the lengths of lateral roots that initiated in the absence of NO_3^- were similar among all treatments (Fig. 3.6b). These results on maize are consistent with those on *Arabidopsis* in which exposure of root patches to NO_3^- stimulated lateral root elongation in the patch (Zhang & Forde, 1998; Zhang *et al.*, 1999; Linkohr *et al.*, 2002). We also found in maize that exposure to the higher osmotic treatments (5.0 mM KCl or 5.0 mM KNO_3) enhanced lateral root density in zones initiated before the treatments began (Fig. 3.6c). All the lateral root primordia eventually emerged (data not shown). Seminal root lengths were similar under all treatments (Fig. 3.6a). Altogether, these observations – higher lateral root densities, all lateral roots emerge and similar seminal root lengths – imply that higher osmotic strengths stimulated maize lateral root initiation.

We also compared instantaneous elongation of maize seminal roots at (a) pH 6.5 and 5.6, a pH range found in the soils around Davis, California (DeClerck & Singer, 2003); (b) in the presence or absence of exogenous NO_3^- or

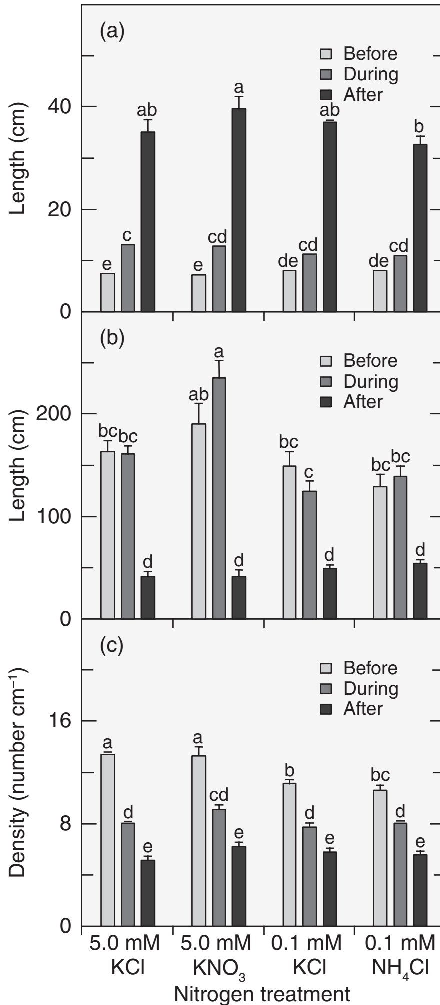


Figure 3.6 (a) Seminal root length, (b) lateral root length and (c) lateral root density (number per unit length of seminal root) in maize plants that were treated for 48 hours with either 5 mM KCl, 5 mM KNO_3, 0.1 mM KCl or 0.1 mM NH_4Cl. Before, during and after refer to the root regions of the seminal root that initiated before, during and after the treatments. Shown are the mean ± SE for 12 plants (Bloom *et al.*, 2002a).

NH_4^+; and (c) in the presence or absence of KCl (Bloom *et al.*, 2006). Elongation in a nitrogen-free nutrient solution was slightly faster at pH 6.5 than at pH 5.6 (Fig. 3.7). Exposure to 100 μM NO_3^- or 100 μM NH_4^+ at pH 6.5 stimulated elongation by 14 or 29%, respectively, in comparison to the nitrogen-free solution at the same pH (Fig. 3.7). The addition of 68 mOsm KCl had little effect on elongation in the nitrogen-free solutions, but eliminated the stimulation by NO_3^-, not by NH_4^+ (Fig. 3.7).

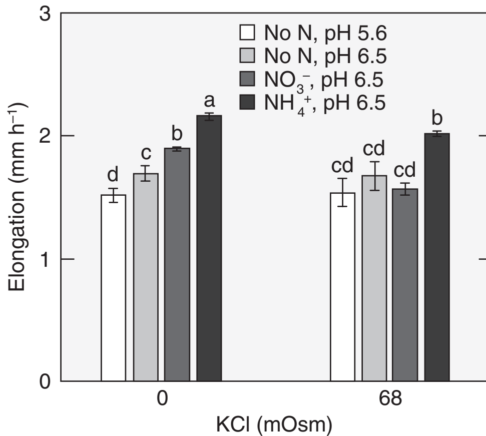


Figure 3.7 Root extension rates for 3-day maize seedlings. The root was exposed to 1 mM $CaSO_4$, 200 μM KH_2PO_4, and either 100 μM KNO_3 (NO_3^-), 100 μM $NH_4H_2PO_4$ (NH_4^+) or no nitrogen (no N). The solutions were maintained (mean ± range) at pH 5.6 ± 0.2 or pH 6.5 ± 0.2 and at the two different osmotic levels (0 or 68 mOsm KCl). Shown are the mean ± SE (six different plants for each of the four nitrogen treatments; each plant was exposed to both osmotic levels). Bars with the same letters did not differ significantly ($P > 0.05$) (Bloom *et al.*, 2006).

These results demonstrate that acidification of the rhizosphere did not promote root elongation of intact maize seedlings (Fig. 3.7); therefore, acid growth of roots was not evident (Bloom *et al.*, 2006). By contrast, the presence of exogenous inorganic nitrogen significantly enhanced elongation (Fig. 3.7), even though the seedlings had ample nitrogen reserves in the caryopse to support growth for several more days (Bloom *et al.*, 2002a). The meristem and transition zones (Baluska *et al.*, 1996) near the root apex differ from more mature root zones in that they lack fully differentiated phloem tissue. Consequently, import of carbon or nitrogen from more mature tissues is relatively slow because it depends on symplastic diffusion (Bret-Harte & Silk, 1994b). For example, little of the NO_3^- absorbed in the maturation zone moves towards the apex (Siebrecht *et al.*, 1995; Walter *et al.*, 2003). The nitrogen thus required for cell division and isotropic cell expansion may derive primarily from nitrogen that the apical zones themselves absorb and assimilate.

The presence of NH_4^+ stimulated root elongation (Fig. 3.7) and accumulation of root biomass (Fig. 3.8) to a greater extent than that of NO_3^-. This is consistent with other studies (Bloom *et al.*, 1993) and may reflect that assimilation of NH_4^+ to glutamine consumes the equivalent of about 2 ATPs per NH_4^+, whereas assimilation of NO_3^- to glutamine consumes the equivalent of about 12 ATPs per NO_3^- as discussed above. In the carbohydrate-limited apical meristem (Bret-Harte & Silk, 1994a), the lower energy requirement of NH_4^+ assimilation may permit root apical cells to maintain higher elongation rates and to accumulate more biomass.

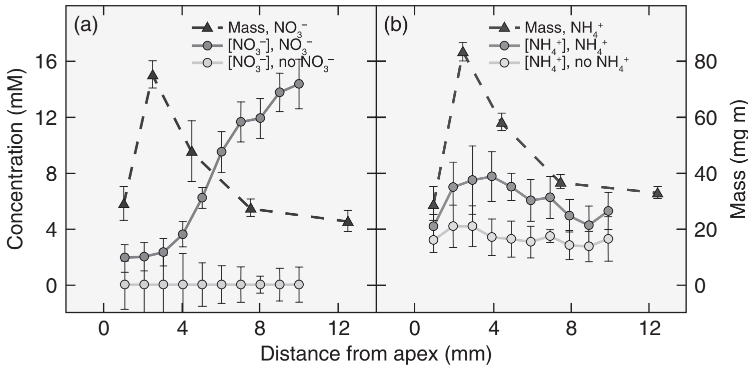


Figure 3.8 Mass of root segments (mg dry mass m^{-1} length) and tissues concentrations of NO_3^- or NH_4^+ ([NO_3^-] or [NH_4^+] in mM based on the per segment water volume) at various distances from the apex of a maize seminal root for plants receiving nutrient solutions that contained either 100 μM NO_3^- (NO_3^-), 100 μM NH_4^+ (NH_4^+) or nitrogen-free (no N) for 18–24 hours. Given are the means ± SE (three to six different plants of each treatment).

Increasing the osmotic potential of the nutrient solution (adding 68 mOsm KCl) had no effect in the treatments devoid of nitrogen ('no N'), but depressed root elongation under NO_3^- and NH_4^+ nutrition (Fig. 3.7). In maize, apical zones of the root rapidly absorbed NO_3^- and NH_4^+ (Taylor & Bloom, 1998). A portion of the NO_3^- absorbed remained as free NO_3^- within the apical zones (Fig. 3.8a), providing a metabolically benign osmolyte (up to 29 mOsm) to support expansion (Bloom, 1996, 1997a; McIntyre, 2001). By contrast, most of the NH_4^+ absorbed promptly disappeared from the tissues (Fig. 3.8b) as the cells presumably assimilated it into amino acids (Bloom *et al.*, 2002a). Some of these amino acids may also serve as metabolically benign osmolytes to support cell expansion in the elongation zone (Rhodes *et al.*, 2002). The addition of 68 mOsm KCl to the nutrient solution depressed root elongation possibly because it counteracted the osmotic effects of the stored NO_3^- and amino acids.

Enhanced root elongation in the presence of exogenous NO_3^- or NH_4^+ is an appropriate response with ecological implications (Bloom, 1997a). To compete successfully with micro-organisms for soil nitrogen, roots proliferate in soil regions that are nitrogen-rich (Hackett, 1972; Drew, 1975; Grime *et al.*, 1986; Sattelmacher & Thoms, 1989; Bingham *et al.*, 1997; Robinson *et al.*, 1999; Zhang *et al.*, 1999). Having root elongation enhanced by exogenous inorganic nitrogen would provide a mechanism for this proliferation.

3.5 Future of plant nitrogen

Atmospheric CO_2 concentrations have increased from about 280 to 380 μmol/mol since 1800 and will reach between 530 and 970 μmol/mol by the end of the century (IPCC, 2007). Crop plants, through their photosynthetic

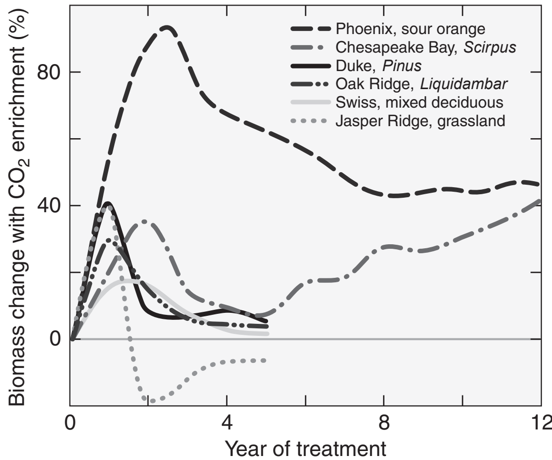


Figure 3.9 Differences in biomass over time (years) between plants grown at elevated (≈567 ppm) and ambient (≈365 ppm) CO_2 atmospheres in free-air CO_2 enrichment (FACE) plots (Dukes *et al.*, 2005; Korner, 2006) and open top chambers (Rasse *et al.*, 2005; Kimball *et al.*, 2007).

conversion of atmospheric CO_2 into carbohydrates and other organic compounds, have the potential to mitigate rising CO_2 levels. The extent to which they will do so remains uncertain, however, because of the wide range in plant responses to CO_2 enrichment.

We believe the complexity of plant responses to CO_2 enrichment derives in part from the dependence of NO_3^- assimilation on photorespiration in C_3 plants and the variation of NO_3^- and NH_4^+ availability over seasons, years, locations and species. Sour orange trees (*Citrus aurantium* L.) fertilized with superfluous amounts of nitrogen showed only a small decline in leaf nitrogen (Idso *et al.*, 1996) and primary productivity (Fig. 3.9). Net primary productivity diminished under CO_2 enrichment in an annual California grassland (Jasper Ridge) for which NO_3^- was the predominant nitrogen source (Fig. 3.9), presumably because CO_2 enrichment inhibited NO_3^- assimilation and organic nitrogen became limiting. By contrast, NH_4^+ is the major form of nitrogen available to plants in marshes because wet, anaerobic soils promote denitrification and NO_3^- leaching. CO_2 enrichment consistently stimulated photosynthesis and growth of *Scirpus olneyi*, the dominant C_3 plant in the Chesapeake Bay marsh (Fig. 3.9), with little change in plant nitrogen concentrations (Erickson *et al.*, 2007). In the future, C_3 species that rely heavily on NO_3^- as a nitrogen source will most likely be at a disadvantage in comparison to those that rely heavily on NH_4^+.

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Chapter 4

TRANSPORT SYSTEMS FOR NO_3^- AND NH_4^+

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Abstract: *Arabidopsis* transporter families for both nitrate and ammonium have been identified and characterized in some detail. Gene knockout studies have been very useful for identifying the physiological function of specific genes in *Arabidopsis*. In the post-genomic era, the identification of genes with orthologous transport functions in other plant species is now relatively easy. However, several key areas of nitrogen transport still require a much better understanding in the model plant *Arabidopsis*, and some of these are described in this chapter.

Keywords: ammonium transport; nitrate transport; nitrogen efflux; regulation of nitrogen transport systems

4.1 Nitrogen forms available to plants

Nitrogen (N) is usually acquired from the soil by plant roots, and this key resource is taken up from the soil solution as nitrate and ammonium or amino acids. Small amounts of nitrogen can be directly acquired from the surface of the leaf when it is deposited from the atmosphere on the leaf surface, although this source is usually only a very minor supply. Some plants can directly acquire gaseous N from the air by symbiotic interactions with micro-organisms. In legumes, nitrogen is fixed in the root nodules, but this process can also occur in less specialized structures, and bacteria living either inside plants or on the root surface can capture N for subsequent use by plants. These sources of N become very important under conditions of low supply

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when the ability of plants to exploit these interactions with bacteria and fungi offers considerable competitive advantage.

The available concentrations of N in the soil can vary greatly from μM to mM ranges and can depend on a large range of soil physico-chemical properties and environmental conditions such as water supply and temperature (Miller *et al.*, 2007). Soil microbial activity determines the form and the concentration of N available. In agriculture, N fertilizer is applied to soil either directly as nitrate or ammonium or more usually as urea. In aerobic temperate soils, microbial activity quickly converts all forms of N to nitrate, and so this is often the most abundant source for crop plants. In more extreme environments, when soil temperature is either hotter or cooler than temperate systems, ammonium and amino acid availability increases (Miller & Cramer, 2004). The concentration of free amino acids in the bulk soil solution ranges from 0.1 to 50 mM, with the greatest concentrations in the surface horizons of soils rich in organic matter (Jones *et al.*, 2002). Although in agricultural soils the concentrations generally range between 1 and 100 μM (Jones *et al.*, 2002), much of this organic N form is derived from the microbial breakdown of plant matter as the concentration of amino acids in tissues is typically 1–10 mM, making this an important source of organic N for the soil. The utilization of this amino source of N has been nicely demonstrated using mutant plants lacking the amino acid uptake system at the root (Svennerstam *et al.*, 2007).

4.2 Nitrogen transport steps and mechanisms

Nitrogen uptake into root and leaf cells requires influx across the plasma membrane. Most soil concentrations of nitrate and ammonium require uptake that is energetically uphill and uses cotransport with protons and the energy for this step provided by pH gradient across the plasma membrane. Once inside the cell, nitrate and ammonium transport is also required in subcellular organelles, such as the chloroplast and vacuole. Nitrate can be stored in the vacuole where it is important for cation charge balance and as an osmoticum for cell expansion or growth. Concentrations may exceed 2% fresh weight (17–24% dry weight) in extreme physiological conditions (Hewitt *et al.*, 1979). Vacuolar nitrate is also a store of N and it can be remobilized to maintain the supply needed for growth (Van Der Leij *et al.*, 1998). For the vacuolar nitrate transport, protons are exchanged in the opposite direction and the energy for this antiport mechanism is provided by the inside acidic gradient of the vacuole (see Fig. 4.1). Nitrate is reduced to nitrite by nitrate reductase in the cytoplasm; the next step in assimilation occurs in the plastid or chloroplast, and nitrite transporters are present in the envelope of the organelles. This transport step may be proton-coupled to ensure that low concentrations of nitrite are maintained in the cytoplasm. Nitrite is a powerful oxidizing agent and is likely to be toxic if accumulated in the cytoplasm,

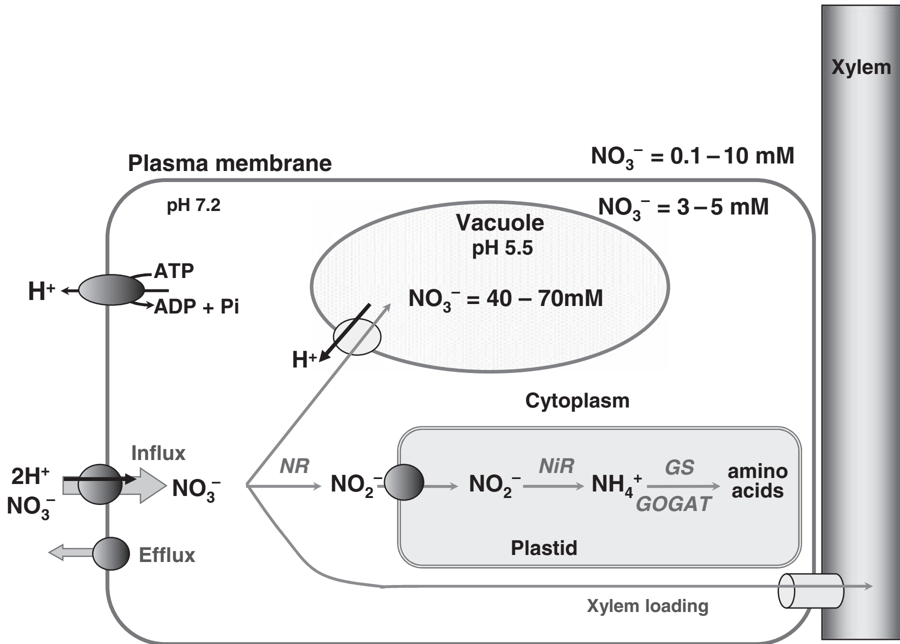

Figure 4.1 Nitrate transport steps and their mechanisms at cellular membranes within the plant. *Note*: For simplification the ATPase and PPase proton pumps in the tonoplast that acidify the vacuole and help to maintain cytosolic pH are not shown. Dark black arrows represent proton fluxes, and some typical values are given for the cellular compartmental concentrations of these ions.

so the plastid transport system is likely to have a high affinity for the ion. Assimilation continues with the reduction of nitrite to NH_4^+ in the plastid and then continues by linking to carbon compounds for the synthesis of amino acids.

NH_4^+ is at equilibrium with NH_3 and the relative amounts of each form depend on the pH. The equilibrium constant for the reaction is 5.7×10^{-10}, so at pH 7, the relative ratio of NH_4^+ to NH_3 is approximately 1000 to 1. Both forms can cross the membrane, but the mechanism is a matter for some debate. NH_4^+ can cross in a channel-type mechanism that is driven by the electrochemical gradient of the ion (Ludewig *et al.*, 2003). The uncharged form, NH_3, can also cross the membrane, and this is described further in relation to the accumulation of NH_4^+ in the vacuole. The pH dependence of the different forms means that the NH_3 form predominates in more acidic compartments, such as the vacuole. Vacuolar concentrations of NH_4^+ are much lower than those measured for nitrate, usually in the low mM range, but reliable estimates for the cytoplasm are difficult to obtain (reviewed by Miller *et al.*, 2001).

4.3 *Arabidopsis* as a model

Ammonium and nitrate transport in *Arabidopsis* is one of the best characterized systems both genetically and physiologically, and it seems to be a valid model for many other species. There are a few aspects of the plant N physiology that differ from some species. In general, the main physiological differences between model plants such as *Arabidopsis* and crops result from the growth conditions. Crop species are grown in fields as a population of plants that are competing for N alongside other resources. For example, leaf shading can greatly influence light capture and photosynthetic efficiency. Therefore, the rules for the allocation of carbon to the root system and the regulation of N uptake might be very different from *Arabidopsis*. The time course of N and carbon partitioning and the source/sink strength is likely to be different for each species. Therefore, the allocation of N and carbon to new sinks, such as seeds, will be different for each crop species.

Arabidopsis is most closely related to *Brassica* crop species such as oilseed rape. When compared with other crops, *Brassica* species and especially winter oilseed rape (*Brassica napus*) are well-known for their high nitrate uptake ability at the vegetative stage. Compared to most other crops, *B. napus* needs substantially higher amounts of N fertilizer (typically 180–220 kg/ha). The nitrogen uptake systems have been described for *B. napus*, and their general feature of regulation at the vegetative stage is similar to *Arabidopsis*. The high level of similarities between the genome sequences of *Brassica* species allowed the identification of *B. napus* nitrate transporter genes using *Arabidopsis* sequences (Faure-Rabasse *et al.*, 2002).

B. napus plants lose a significant amount of N from early leaf shedding (Rossato *et al.*, 2002), and subsequent breakdown can release a high amount of N that is potentially available to be taken up. The ability of oilseed rape to absorb this N late in the plant growth cycle is still under debate. In hydroponic conditions, N uptake after flowering has been shown to be strongly down-regulated (Beuve *et al.*, 2004), but in field experiments, up to 30% of the total N absorbed is taken up during the seed-filling stage (Malagoli *et al.*, 2005). The regulation of root nitrate uptake in *Arabidopsis* at the reproductive stage is currently under investigation to highlight similarities or differences between the two species.

4.4 Ammonium transporters

The high-affinity uptake of ammonium by plants is mediated by membrane proteins of the AMT1 and AMT2 subfamilies (Loqué & von Wirén, 2004). These proteins were first characterized by expression in yeast cells, but later their function has been confirmed by the characterization of *Arabidopsis* mutants deficient in the proteins. The membrane proteins can function as homo- or hetero-oligomeric complexes, and they mediate the uniport of

NH_4^+ (Ludewig *et al.*, 2003). The accumulation of NH_4^+ in tissue can be toxic, and to avoid this problem a rapid shut-off mechanism is required. It was shown that the cytosolic carboxy terminus of an *Arabidopsis* AMT transporter was an allosteric regulator of function (Loqué *et al.*, 2007). Mutations in this C-terminal domain, which is conserved between bacteria, fungi and plants, led to loss of transport activity. In plants, the AMTs mediate uptake at the cell plasma membrane, where they function in N acquisition from the soil, but also in the recovery of leaked NH_4^+ which can efflux from the cell as ammonia under certain conditions.

4.4.1 Ammonium uptake by roots

Like nitrate, the uptake of NH_4^+ at the plasma membrane by plant root cells can be decreased by increasing the external supply of amino acids and NO_3^- (e.g. Lee *et al.*, 1992; Rawat *et al.*, 1999). In most agricultural soils, plant roots are usually exposed to more NO_3^- than NH_4^+ (Miller *et al.*, 2007). Ammonium uptake is mainly mediated by the AMT family of transporters, and the regulation of this transport can occur by several different mechanisms. As this topic has been carefully reviewed in detail (Loqué & von Wirén, 2004), we will only give a brief overview of some points that are relevant to transport and N status. In *Arabidopsis*, the AMT family is largely responsible for root uptake of ammonium. By using T-DNA insertion *Arabidopsis* plants, it was shown that AtAMT1;1 mediates 30% of the total ammonium uptake capacity (Kaiser *et al.*, 2002). Similarly, a gene knockout line AtAMT1;3 also showed a 30% decrease in ammonium uptake, and when double lines, deficient in both AMTs, were compared with wild-type plants, the changes were additive (Loqué *et al.*, 2006).

Feeding roots with glutamine and the use of a glutamine synthetase inhibitor have led to a model proposing that glutamine in the cell altered transcription while cytosolic concentrations of NH_4^+ may post-translationally regulate one AMT gene (Rawat *et al.*, 1999). More generally, regulation of uptake can occur at the mRNA level and AMT transcripts are strongly dependent on the N status of the plant, but in *Arabidopsis* the pattern is different for family members. Some AMTs increase expression earlier during N deficiency, while others can increase after more prolonged starvation (Loqué & von Wirén, 2004). Split-root experiments have suggested that the local root, rather than whole plant, N status regulates the expression of an NH_4^+ transporter (Gansel *et al.*, 2001). This result was different for an NO_3^- transporter where the N status of the whole plant was important (Gansel *et al.*, 2001). The expression of transporters for both NH_4^+ and NO_3^- can be stimulated by photosynthesis, and this occurs by changes in the availability of sugars (Lejay *et al.*, 2003).

Tobacco plants with 35S-driven expression of an *Arabidopsis* AMT showed a 30% increase in root uptake of NH_4^+ in hydroponics compared to wild-type plants (Yuan *et al.*, 2007b). However, on soil supplemented with NH_4^+ as an

N source, these plants showed neither growth nor N acquisition differences from wild-type plants (Yuan *et al.*, 2007b). Despite expression being driven by the 35S promoter in these tobacco plants, the steady-state transcripts for *AtAMT1;1* were not constitutive, changing with N status of the plants and decreasing after NO_3^- or NH_4^+ addition to N-deficient roots. This result suggests that the N status of a plant may directly influence mRNA turnover in plants, and this may provide another regulatory mechanism for NH_4^+ uptake. This result seems to contrast with that for NO_3^- transporter transcripts. In *Nicotiana plumbaginifolia* the expression of *NpNRT2.1* driven by *rolD* or the *35S* promoters was still high even after treatment with 10 mM NO_3^-, when the WT endogenous gene was repressed (Fraisier *et al.*, 2000). In *Arabidopsis* the post-translational regulation of AtAMT1;1 provides a rapid way of regulating NH_4^+ transport activity that can be linked to N supply by the phosphorylation of the C terminus (Loqué *et al.*, 2007). Furthermore, the differing affinities for NH_4^+ together with the spatial arrangement of AMT family members provide a coordinated system for uptake in the root (Yuan *et al.*, 2007a). AtAMT1;1 and 1;3 are located in the plasma membrane of the rhizodermal cell layer. AtAMT1;2 was expressed in the plasma membrane of endodermis and root cortex cells and therefore mediates the uptake of ammonium entering the root via an apoplasmic transport route. While AtAMT1;5 was expressed in N-deficient rhizodermal and root hair cells, it also provides a secondary route for entry. Using gene knockout mutants, the *in planta* NH_4^+ influx substrate affinities of AMT1;1,1;2,1;3 and AMT1;5 were measured as 50, 234, 61 and 4.5 μM, respectively (Yuan *et al.*, 2007a). The higher affinity for NH_4^+ of AtAMT1;5 fits with the idea that it is expressed under N deficiency. Together this arrangement of the *Arabidopsis* AMTs provides an efficient uptake system for NH_4^+ in roots.

4.4.2 Vacuolar ammonia transport

Aquaporins have been implicated in ammonia transport (Niemietz & Tyerman, 2000; Jahn *et al.*, 2004). The expression of two *Arabidopsis* aquaporins in yeast cells was found to give tolerance to methylammonium, a toxic analogue of NH_4^+ (Loqué *et al.*, 2005). These aquaporins belong to the tonoplast intrinsic protein (TIP) subfamily and when expressed in *Xenopus* oocytes they increased ^{14}C-methylammonium accumulation with increasing external pH relative to controls. The *Arabidopsis* TIP-mediated methylammonium detoxification in yeast depended on a functional vacuole and the subcellular localization of the GFP-fusion proteins on the tonoplast *in planta.* Taken together, these results suggested these tonoplast aquaporins can mediate the vacuolar transport of ammonia. The transcript levels of both AtTIPs were influenced by nitrogen supply, but did not follow those of the N-derepressed ammonium transporter gene AtAMT1;1. However, transgenic *Arabidopsis* plants overexpressing one of these tonoplast aquaporins, AtTIP2;1, did not show altered NH_4^+ accumulation in roots after NH_4^+ supply (Loqué *et al.*, 2005).

As discussed above, the equilibrium between ammonia and NH_4^+ results in the passive accumulation within more acid cellular compartments, such as the vacuole. Decreasing vacuolar pH to give increased acid trapping has even been proposed as a route for genetically engineering increased N storage in crops to improve N use efficiency (Wood *et al.*, 2006).

4.4.3 Transport of NH_4^+ in the peribacteroid membrane

Soil bacteria of the various species of *Rhizobium* infect roots and induce the formation of nodules. Within certain cells of the nodules, the N_2-fixing bacteria differentiate into bacteroids that are surrounded by a plant-derived peribacteroid membrane (PBM) forming an organelle-like structure, the symbiosome. Like the tonoplast surrounding the cellular vacuole, the PBM surrounds an acidic compartment. The PBM controls nutrient exchange between the bacteroids and the host cell that is mainly fixed nitrogen in the form of NH_3 or NH_4^+ from the bacteroids and reduced carbon from the plant. In legumes, a membrane that has a high density of NH_4^+/NH_3 transport that surrounds an acidic compartment is the peribacteroid membrane. The transport of NH_3 and NH_4^+ across the PBM has been shown to be mediated by both aquaporins (Niemietz & Tyerman, 2000) and specific channels that were blocked by Mg^{2+} on the symbiosome lumen side (Obermeyer & Tyerman, 2005).

4.5 Nitrate transporters

Many NO_3^- transporters have been cloned from a variety of species, and several different families have been identified. The best characterized are the NRT1 and NRT2 families, and the *Arabidopsis* genome contains 53 NRT1 and 7 NRT2 members (Williams & Miller, 2001). These proteins typically have 10–12 predicted trans-membrane domains, and the cellular location and transport mechanism have been characterized for only a few family members. A further complication for the NRT1 family is that they belong to a larger family of peptide transporters, the POT, or proton-dependent oligopeptide transport family which is also known as the PTR or peptide transport family (Paulsen & Skurray, 1994). Mammalian members of this family can transport peptides of varying sizes, and one plant NRT1 protein can transport nitrate, peptides and some basic amino acids (Zhou *et al.*, 1998). The NRT2 family belongs to a larger group of transporters, the nitrate/nitrite permeases (NNP), and this name reflects their ability to transport both these substrates (Forde, 2000; Orsel *et al.*, 2002). Some members of the NRT2 family require a second gene product for functional activity, and this gene product is required for a protein–protein interaction that seems to assist in targeting the NRT2 protein into the plasma membrane (Orsel *et al.*, 2006). This second protein is much smaller, typically only 200 amino acids long and containing only one predicted transmembrane domain (Tong *et al.*, 2005). They have

been confusingly named NRT3 (Okamoto *et al.*, 2006), but do not themselves appear to directly transport nitrate (Tong *et al.*, 2005).

Other families of nitrate transporters include the CLCs (De Angeli *et al.*, 2006) and nitrate efflux proteins (Segonzac *et al.*, 2007); the former mediate nitrate transport into the vacuole, while the latter are involved in pH regulation and nitrate efflux from cells.

4.5.1 Nitrate uptake by roots

Measurements of NO_3^- uptake by the roots of many different types of plants have led to the conclusion that plants have developed three types of transport system to cope with the variations in NO_3^- concentrations in cultivated soils (Crawford & Glass, 1998). Two saturable high-affinity transport systems (HATS) are able to take up NO_3^- at low external concentrations (1 μM to 1 mM). The constitutive system (cHATS) is available even when plants have not been previously supplied with NO_3^-. The inducible system (iHATS) is stimulated by NO_3^- in the external medium. The low-affinity transport system (LATS) displays linear kinetics, and its contribution to NO_3^- uptake becomes significant at external NO_3^- concentrations above 1 mM (Crawford & Glass, 1998). Although in principle both types of HATS can contribute to nitrate uptake at external concentrations above 1 mM, their transport activity is saturated and expression is down-regulated such that they actually contribute marginally to N acquisition. In *Arabidopsis*, the borders between these various physiological measures of NO_3^- uptake are vague and the explanation for this may be due to experimental design. For example, these parameters may depend on the plant developmental stage, time of day and the ecotype used. The use of *Arabidopsis* mutants that have disruptions in specific transporter genes has been a powerful tool for the identification of key transporters involved in uptake.

4.5.2 Low-affinity nitrate transport

The concentration of NO_3^- that is available in the soil is chiefly found in the mM range, and we have much less knowledge of the molecular identity of LATS when compared with HATS. AtNRT1.1 (formerly called CHL1) was originally identified as a contributor to LATS (Tsay *et al.*, 1993) as deletion mutants only showed a low-affinity uptake phenotype when growing on a mixed NO_3^- and NH_4^+ supply (Touraine & Glass, 1997). This result led to the suggestion that there may be a second LATS that can only compensate when plants are grown with NO_3^- as the only N source. A later comparison between gene expression levels and LATS activity showed that AtNRT1.1 was mainly responsible for influx at 5 mM external concentrations of NO_3^- (Okamoto *et al.*, 2003). However, LATS is assumed to be constitutively present in roots, and *AtNRT1.1* expression is NO_3^--inducible. Furthermore, the expression of *AtNRT1.1* occurs in very specific tissues at primary and lateral

root tips and in stomata where it has a role in water stress responses (Guo *et al.*, 2003). A further complication arises from the fact that the protein was actually described as being dual affinity, with a phosphorylation switch between high- and low-affinity ranges of nitrate uptake (Liu & Tsay, 2003). In contrast, AtNRT1.2 is constitutively expressed in root epidermal cells and has a K_m for NO_3^- about 6 mM in oocytes (Huang *et al.*, 1999). However, *Arabidopsis* antisense lines of *AtNRT1.2* were characterized by NO_3^- depletion assays and electrophysiology that showed a 50–70% decrease in LATS, but these changes did not correlate with the drop in expression across the lines (Huang *et al.*, 1999). Gene deletion lines of *AtNRT1.1* were used for comparison in these experiments, and these also showed a 45% decrease in LATS. Better methods for characterizing the NO_3^- influx, such as $^{15}NO_3^-$ influx, should be used to check LATS in these gene-disrupted lines. Taken together, these data seem to suggest a complicated role for both AtNRT1.1 and ATNRT1.2 in LATS, but this result requires re-examination especially as *AtNRT1.1* is not strongly expressed in the epidermis and cortex of roots (Guo *et al.*, 2001). *AtNRT1.4* has a very specific pattern of expression in the leaf petiole where it has a role in NO_3^- accumulation within these tissues (Chiu *et al.*, 2004). Expression of *AtNRT1.3* was NO_3^--induced in the leaf but repressed in the root, and does not seem to be a significant contributor to LATS (Okamoto *et al.*, 2003). Another member of the NRT1 family, AtNRT1.5, was shown to be involved in pH-sensitive long-distance transfer of nitrate from the root to the shoot (Lin *et al.*, 2008). There are many other LATS candidate genes among the NRT1/PTR family. One aspect of the thermodynamics of LATS is the fact that the transport system need not always be coupled to two protons (Miller & Smith, 1996). Therefore, the components of LATS may belong to another gene family that is as yet uncharacterized. Clues to identifying the genes responsible for LATS may come from the fact that it is a constitutively expressed system. However, this may also mean that it may be of fundamental importance to plants and gene-disrupted mutants may be lethal. The molecular identity of LATS remains a key target for future research especially for uptake by crops.

4.5.3 High-affinity nitrate transport

Three genes have been identified as being responsible for *Arabidopsis* root uptake in the HATS range are *AtNRT2.1; AtNRT2.2* and *AtNAR2.1* (also named *AtNRT2.3*). *Arabidopsis* mutants with impaired expression of *AtNRT2.1* and *ATNRT2.2* genes were shown to be defective in HATS activity (Filleur *et al.*, 2001; Orsel *et al.*, 2004). These two genes lie end to end in the *Arabidopsis* genome and encode very similar proteins, 90% identity (Orsel *et al.*, 2002). Interestingly, parallel gene pairs of NRT2s that are also located closely within the genome are found in other species, for example *Chlamydomonas* (Quesada *et al.*, 1994) and rice (Araki & Hasegawa, 2006), and this may be a more general pattern. In rice the two sequences appear to be identical, but the

untranscribed flanking sequences result in different patterns of gene expression (Araki & Hasegawa, 2006). This information may suggest that functionally homologous genes encoding major components of HATS may have evolved early in plant development by a gene duplication event. Another *Arabidopsis* mutant has been described that is only disrupted in expression of *AtNRT2.1*, and this has been used to dissect the relative contributions of these two genes to iHATS and cHATS and to define *AtNRT2.1* as the major contributor to both systems (Li *et al.*, 2007) and in accordance with their relative expression level (Filleur *et al.*, 2001). A mutant in *AtNAR2.1* (*AtNRT2.3*) has also been identified, and these plants have shown the important contribution of this gene to HATS (Okamoto *et al.*, 2006; Orsel *et al.*, 2006). These plants have lost even more of their HATS than the *AtNRT2.1/2.2* mutants, suggesting that there may be some nitrate transporters that may make a minor contribution to the uptake and have a requirement for AtNAR2.1 for function (Orsel *et al.*, 2006). The role of AtNAR2.1 in the correct targeting of GFP-tagged AtNRT2.1 transporter protein to the plasma membrane has been identified (Wirth *et al.*, 2007).

4.5.4 Feedback and regulation of nitrate transport system

The uptake of NO_3^- is subject to negative feedback regulation that depends on the N status of the plant. The uptake of both NO_3^- and ammonium is increased by N deficiency (Lee & Rudge, 1986). There are several different routes whereby NO_3^- uptake can be changed, and these include both changes in transporter gene expression and post-translational mechanisms (see review, Miller *et al.*, 2007).

The N status of plants may be indicated by the pool sizes of specific N-containing molecules and ions, including ammonium and NO_3^- (Fig. 4.1). For example, supplying amino acids to roots inhibits NO_3^- uptake in many different types of plants, including trees (Dluzniewska *et al.*, 2006). The expression of inducible high-affinity NO_3^- transporters can be effectively inhibited by glutamine (Krapp *et al.*, 1998; Vidmar *et al.*, 2000). Transporter transcript abundance and NO_3^--induced influx were decreased simultaneously in the root tissue treated with exogenously applied amino acids (Vidmar *et al.*, 2000). As amino acids can be interconverted within plant tissues, chemical inhibitors of the conversion steps were used to identify glutamine as being responsible for down-regulating NO_3^- transporter expression (Vidmar *et al.*, 2000). In *B. napus*, a negative correlation was found between either shoot N or NO_3^- contents and NO_3^- uptake rates, but pools of free amino acids in roots did not seem to be involved in the control of root NO_3^- uptake (Lainé *et al.*, 1995). For this species, a positive correlation between γ-aminobutyric acid in the phloem and NO_3^- uptake was reported (Beuve *et al.*, 2004). This result is unusual because a negative feedback system is more common for most plants, although even in *B. napus* treating the roots with amino acids decreased NO_3^- uptake and transporter expression (Beuve *et al.*, 2004).

Nitrate uptake can also be influenced by NH_4^+ supply, and when a mixed N source is supplied to plants, NO_3^- uptake is usually decreased (e.g. Kronzucker *et al.*, 1999). Like amino acids, NH_4^+ requires less energy input by the plant because the N is already in a reduced form bypassing this step in assimilation. However, in contrast to amino acids, there can be some problems for cellular pH regulation associated with NH_4^+ as an N source for the plant (Raven & Smith, 1976) and, at high external concentrations, the energy expended in effluxing this cell toxic ion (Britto *et al.*, 2001).

In *Arabidopsis*, for the regulation of NO_3^- uptake plants can sense specifically a lack of this ion, not an overall N deficiency. The expression of *AtNRT2.1*, the main component of HATS, is known to be induced by NO_3^-, and feedback is repressed by reduced N metabolites. Surprisingly, in media containing high concentrations of ammonium or glutamine, *AtNRT2.1* expression increases when the NO_3^- concentration decreases to a low level (<0.5 mM). This NO_3^--specific response, including the repression of the HATS by high concentrations, is mediated by another transporter AtNRT1.1 (Krouk *et al.*, 2006), and may involve transient changes in the cytoplasmic NO_3^- pool (Fan *et al.*, 2006).

4.5.5 Sensing role of nitrate transporters

Root growth assays have become a powerful route for dissecting the contribution of nitrate transporter proteins in sensing nitrogen availability. The architecture of roots has been successfully used as an assay to dissect the NO_3^- signalling pathway (see Chapter 7). In *Arabidopsis*, two of the nitrate transporters, AtNRT1.1 and AtNRT2.1, are particularly implicated in the way in which roots sense nitrate availability (see Remans *et al.*, 2006a, 2006b). Interestingly, the root response to glutamate appears to also be mediated by AtNRT1.1, suggesting a common mechanism for the sensing of both inorganic and organic N forms (Walch-Liu & Forde, 2008).

4.5.6 Vacuolar nitrate transport

Nitrate storage in the vacuole is important for osmotic balance and as an N reserve. Tissue levels are indicators of N status, and this is the basis of tissue testing for crops. Land plants accumulate NO_3^- in the vacuole, but aquatic plants may have either lost this ability or never developed it. Giant algal cells did not accumulate NO_3^- in the vacuole above passive transport levels even after growing in high NO_3^- concentrations for many months (Miller & Zhen, 1991). Seven members of the CLC gene family have been identified in the *Arabidopsis* genome (De Angeli *et al.*, 2006), and disruption of one of these genes was shown to alter the accumulation of NO_3^- in leaf tissues (Geelen *et al.*, 2000). In mammals, members of the CLC family were localized in endomembranes, including the mitochondria. Studying the function of mammalian CLCs has been made easier by the fact that the proteins

have been characterized by expression in *Xenopus* oocytes, but this approach has been unsuccessful for plant homologues. GFP tagging of the proteins in *Arabidopsis* and rice has localized them to the tonoplast where they are implicated in the storage and remobilization of NO_3^- and chloride in the vacuole (De Angeli *et al*., 2006; Nakamura *et al*., 2006). Much earlier studies with isolated vacuoles (Schumaker & Sze, 1987) and intracellular measurements of pH and NO_3^- gradients had used thermodynamic calculations to predict an antiport mechanism for NO_3^- accumulation in the vacuole (Miller & Smith, 1992). These results are consistent with the new finding that AtCLCa is able to accumulate specifically nitrate in the vacuole and behaves as a $2NO_3^-/H^+$ antiporter (De Angeli *et al*., 2006). This family of transporters is important for further studies to understand and manipulate N use efficiency in crops. Nitrate storage pools in the leaves and stems of crop plants are important for several quite different reasons. Firstly, increased storage in tissue enables overwintered crops in the mopping up of soil NO_3^- that would otherwise cause environmental damage through leaching in rainwater. Secondly, when leafy vegetables are eaten, there is medical evidence to suggest that it is beneficial to health to decrease tissue NO_3^- concentrations. This family of transporters may have a role in salt tolerance as these genes can also transport chloride, resulting in accumulation in the vacuole to avoid any possible harmful effects of the anion in the cytoplasm. There is good evidence that AtCLCa is not solely responsible for vacuolar accumulation of nitrate. Although the mutant deficient in the AtCLCa gene is deficient in tissue nitrate accumulation, the vacuolar concentrations of nitrate relative to wild type would suggest that there are other tonoplast transporters that contribute to loading nitrate into the vacuole (Geelen *et al*., 2000).

Recently, one member of the NRT2 family was found localized to the vacuolar membrane, and this transporter was specifically expressed in the developing seed (Chopin *et al*., 2007). Mutant plants deficient in this transporter, AtNRT2.7, did not accumulate nitrate in the seed and showed delayed germination.

4.5.7 Nitrate efflux

In general, NO_3^- efflux systems have been much less studied than influx systems; however, it is known that efflux is protein-mediated, passive, saturable and selective for NO_3^- (e.g. Aslam *et al*., 1996). Aquaporins or more likely anion channels are an obvious route for NO_3^- efflux because they are thermodynamically downhill. However, one member of the NRT2 gene family was demonstrated to have both a proton cotransport and passive mechanism (Zhou *et al*., 2000). The NO_3^- efflux system is under a degree of regulation, induced by NO_3^- (Aslam *et al*., 1996), and it is also proportional to whole tissue NO_3^- concentrations (Teyker *et al*., 1988; Van Der Leij *et al*., 1998). We can predict that the membrane protein mediating NO_3^- efflux must be NO_3^--inducible.

Concentrations of NO_3^- in the xylem sap can be quite high (10–30 mM) especially in plants which transport most of the NO_3^- taken up to the shoot for reduction. Nonetheless, the entry of NO_3^- into the xylem can be mediated by anion channels, assuming cytosolic NO_3^- concentrations and membrane potentials in xylem parenchyma cells similar to those measured in epidermal and cortical cells (Miller & Smith, 1996). Genome analysis has identified several different anion channel families that may fulfil this function. Candidate quickly-activating anion channels have been characterized for barley roots (Kohler *et al*., 2002), but their molecular identity has not yet been determined. Nitrate in the xylem exerts positive feedback on its loading through a change in the voltage dependence of the channel. Interestingly, this effect was specific for NO_3^- and was not found for Cl^-. By transport through this channel, NO_3^- efflux into the xylem can be maintained with high NO_3^- concentrations in the xylem sap, a situation which can occur during the night. There is a clear diurnal change in xylem sap concentrations (Siebrecht *et al*., 2003), related to changes in the transpiration rate. In maize xylem parenchyma cells, the activity of a quickly activating anion channel was altered by changes in water status of plants and addition of abscisic acid to protoplasts inhibited the channel activity (Gilliham & Tester, 2005).

Nitrate efflux is an essential component of the signalling pathway leading to defence responses and hypersensitive cell death in tobacco (Wendehenne *et al*., 2002). Chemicals that inhibit NO_3^- efflux inhibitors reduced and delayed hypersensitive cell death and decreased or completely suppressed the induction of several defence-related genes in tobacco. These results indicated that anion channels are involved intimately in nitrate release from cells and in mediating cell defence responses.

4.6 Plastid transport

Transport across the plastid envelope is important because it provides the linking point between carbon and N assimilation. There must be good coordination between the imports of nitrite and C skeletons into the chloroplast, and these steps provide a possible key site for regulation of the processes. It is interesting to note then that mutants of the PII protein appear to be altered in chloroplast nitrite uptake (Ferrario-Mery *et al*., 2008). PII protein is a nuclear-encoded plastid protein that regulates the activity of a key enzyme of arginine biosynthesis. The NH_4^+ assimilating enzyme, glutamine synthetase, is found in the cytoplasm, chloroplast and even the mitochondria (Taira et al., 2004), and so transport of this ion into the chloroplast may be less important as a site for regulation of C and N assimilation. The genes encoding the route for entry of ammonium into the plastid have not been demonstrated, but they could belong to either the AMT or TIP families already identified. Several candidate genes for the plastid nitrite transporter have been identified. In the unicellular algae, *Chlamydomonas* members of the *NAR1* gene family have a

key role in both C and N transport, with at least one family member able to transport both nitrite and bicarbonate (Mariscal *et al.*, 2006). In cucumber, an NRT1-type transporter named CsNitr1-L has been identified as being a candidate chloroplast nitrite transporter (Sugiura *et al.*, 2007). A GFP-tagged form was present in the isolated inner envelope membrane of chloroplasts, but the transport function was not fully demonstrated. However, the protein seemed to be present in two forms, one of which was truncated by 120 amino acid residues. When expressed in yeast, the truncated protein resulted in lower accumulations of the nitrite in the cells, suggesting it may efflux nitrite. A clear demonstration of function is needed, and plants that are defective in plastidic nitrite transport are likely to have a strong phenotype when grown with only nitrate as the N source.

4.7 Conclusions and future

Many different types of plants have been shown to grow best with a mixed N supply, both in hydroponics and sand culture. The homeostasis of pH is easier in plants supplied with a mixed N supply, and yet experiments to study gene expression have often only used one N form at a particular concentration supplied to roots over the entire length of the root. The temporal and spatial interaction between these N forms in soil is variable, and yet this aspect of root physiology and molecular biology has not been studied. In general, the environmental differences between plants growing on agar in Petri dishes or hydroponic experiments to the soil situation are huge, and the consequences for N acquisition are poorly understood. One challenge for the future will be to adapt our understanding of root behaviour and N acquisition pattern to the variability of the soil environment. A factor that is likely to be important is the spatial distribution of transporters within individual tissues and cells.

Another area where there is a large gap in our understanding is the linking steps between changes in N supply and altered levels of growth effectors such as hormones. For example, although the role of auxins and cytokinins in N root responses is known and transporter proteins such as AtNRT1.1 and AtNRT2.1 are the sensors for changes in nitrate availability at the root surface, the way in which the signalling cascade links these 'receptors' and 'effectors' is unknown. The phosphorylation steps of some transporter proteins (e.g. AtNRT1.1; Liu & Tsay, 2003) seem to be good candidates for playing an important role in bridging these two parts of the root response to N availability.

The transport systems for nitrate and ammonium in *Arabidopsis* are now well characterized, and the use of gene knockout has been particularly useful for identifying the physiological function of specific genes. This information now enables the rapid identification of equivalent genes in other species. The use of polymerase chain reaction (PCR) primers designed for conserved parts of the genes in root tissue can be used to find homologues in other species. One interesting question is how to use and apply this information to

physiology. For example, crop cultivars with different N uptake efficiencies can be compared to identify the key root uptake systems in this important process. This type of comparison is likely to provide molecular information that can be used to identify management strategies and breeding markers to improve this parameter in crops. Another approach may be to learn from the N ecology and physiology of native species (e.g. Henry & Jefferies, 2003). An understanding of the pattern of N uptake in plants that grow in nutrient-poor soils can give important insight into strategies for improving N uptake efficiency by crops.

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Chapter 5

NITRIC OXIDE SYNTHASE-LIKE ACTIVITIES IN PLANTS

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Abstract: Nitric oxide (NO) is a gaseous radical molecule that has long been considered to be a harmful air pollutant produced through anthropogenic activities. The finding in the late 1980s that the gas is synthesized in animal cells by the enzyme nitric oxide synthase (NOS) led to a paradigm shift in bioscience. NO is now recognized as an endogenous signalling molecule involved in diverse physiological processes. Plants and algae also produce NO, but the mechanism of synthesis remains controversial. A number of inhibitor experiments have suggested the presence of NOS-like activities in plants and algae. To date, however, there is no conclusive evidence for the presence of NOS enzymes in these organisms. This chapter provides an overview of plant NOS research, along with a description of possible arginine-dependent signal transduction mechanisms in plants.

Keywords: nitric oxide; nitrogen cycle; plant–microbe interaction; signal transduction; soil bacteria

5.1 Introduction

Nitric oxide (NO or nitrogen monoxide) is a simple gaseous molecule that exhibits astonishing multifunctionality in biological systems. Until recently, bacterial activity in soils was the only known biological source of NO, with

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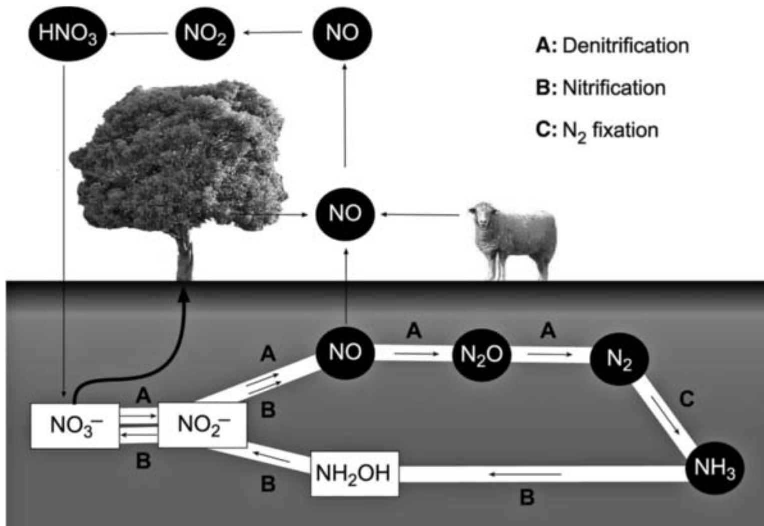


Figure 5.1 NO cycle in the field. Bacterial NO production in soils has been known for many years. Denitrifying bacteria such as *Paracoccus denitrificans* and *Pseudomonas stutzeri* produce NO as a free intermediate of denitrification (route A). Autotrophic nitrifying bacteria such as *Nitrosomonas eutropha* and *Nitrosospira briensis* also emit NO under hypoxic conditions, after flooding, for example (route B). In the troposphere, NO is oxidized by ozone to form NO_2. In urban areas, the oxidation product of NO eventually precipitates as NO_3^- via HNO_3 back into the soil. Recent studies have confirmed that plants and animals possess enzymatic NO production mechanisms. Thus, plants in the field are surrounded by NO from multiple biogenic and anthropogenic sources.

the molecule being considered primarily as a by-product or intermediate of nitrification and denitrification (Fig. 5.1). Although the importance of bacterial NO production in the global nitrogen cycle is now well established (Bothe *et al.*, 2007), for most of the 200 years following its first description by Joseph Priestley, NO had been considered to be physiologically irrelevant in higher organisms. After the discovery of NO-producing enzymes and the corresponding guanosine 3′,5′-cyclic monophosphate (cGMP) signal transduction systems in mammals, our appreciation of NO drastically changed, as seen in the awards of 'Molecule of the Year' by *Science* magazine in 1992 and the Nobel Prize for physiology and medicine in 1998.

The discovery of signalling functions for NO in mammalian cells was an exciting breakthrough for science. This paradigm shift in biology has sparked scientists in other fields to explore NO-dependent signal transduction pathways. This chapter highlights mechanisms for arginine-dependent NO synthesis in plants. Although a number of reports have suggested the presence of arginine-dependent NO-producing activity in plant cells, it remains obscure how plants synthesize NO with arginine (Neill *et al.*, 2008). By reviewing the current stage of our knowledge alongside historical perspectives in the

following discussion, our intention is to highlight some of the prospects for future research in plant NO metabolism in the post-genomic era.

5.2 Lifetime of nitric oxide

The chemical stability of NO under *in vivo* conditions is important, as it is relevant to the biological functions of the gas and its signalling roles. NO is a radical molecule with a lifetime that is shorter than many other known signalling molecules. Moreover, NO degradation is rapid even in the absence of metabolizing enzymes. The degradation of NO can occur by spontaneous oxidation with molecular O_2. This reaction was first described by Joseph Priestley in 1772, and pre-dates his discovery of oxygen and photosynthesis. In his experiments, Priestley produced NO_2 from NO to quantify the O_2 content of air (Yamasaki, 2004):

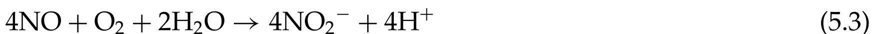
$$2NO + O_2 \rightarrow 2NO_2 \quad (5.1)$$

The half-lifetime of NO ($t_{1/2}$) can be obtained by the following equation:

$$t_{1/2} = \frac{1}{k_2[NO][O_2]} \quad (5.2)$$

The rate constant, k_2, was reported to be $1.4 \times 10^4\ M^{-2}\ s^{-1}$ at 25°C (Hisatsune & Zafonte, 1969). Since the O_2 concentration of air is constant under ambient conditions (~20%), the half-life of NO depends on the NO concentration. For example, the half-life of NO at a concentration of 1 ppm is 56 hours in ambient air (Yoshimura, 1998). While the oxidation of NO by molecular O_2 is relatively slow, the reaction with ozone (O_3) is much faster. Thus, ozone is considered to be a major natural oxidant of atmospheric NO. In the presence of atmospheric concentrations of O_3 (~25 ppm), the half-life of 1 ppm NO is estimated to be about 140 μs (Yoshimura, 1998). These chemical characteristics of NO in the gas phase must be remembered in any consideration of the NO radical as an interorgan or interplant signal.

NO is lipophilic in nature, but like O_2 it can be dissolved in water. The solubility of NO in water is 1.9 mM/atm (Wink *et al.*, 1996). In the aqueous phase, the oxidation of NO can be described by the following simple equation:



$$4NO + O_2 + 2H_2O \rightarrow 4NO_2^- + 4H^+ \quad (5.3)$$

The reaction described in Equation 5.3 is biochemically relevant because biological NO production takes place in the aqueous phase. The Griess assay, one of the methods to quantify NO production, measures nitrite (NO_2^-) that is stoichiometrically produced by the oxidation reaction (Arita *et al.*, 2007). It is important to note that for simplicity, reactive intermediates are not included in Equation 5.3. The nature of many of these intermediates remains uncertain. However, compounds such as NO_2 and dinitrogen trioxide (N_2O_3), which are probably formed in this way, are potentially responsible for NO toxicity in

the aqueous phase (Van Der Vliet *et al.*, 1996). In contrast to the stability of NO in the air, the half-life of NO is short in the aqueous phase, that is, less than 10 seconds (Wink *et al.*, 1996).

Reactive oxygen species (ROS) also determine the lifetime of NO in cells. NO has often been reported to function as an antioxidant (Rubbo & Freeman, 1996). The antioxidant characteristics of NO are ascribed to the direct reaction between NO and ROS, which is also important in assessing the effects of NO in biological systems. In the presence of superoxide (O_2^-), NO can be converted to peroxynitrite ($ONOO^-$), a reactive nitrogen species (RNS):

$$NO + O_2^- \rightarrow ONOO^- \quad (5.4)$$

The rate constant for the reaction between NO and O_2^- means that the rate of the reaction between these two molecules is controlled at near diffusion values (Koppenol, 1998; Squadrito & Pryor, 1998). It is important to note that the rate constant of this reaction is greater than that of the O_2^- disproportionation reaction that is catalysed by superoxide dismutase. $ONOO^-$ has been considered to be a major cytotoxic agent derived from NO (Koppenol, 1998; Squadrito & Pryor, 1998; Wink & Mitchell, 1998; Arteel *et al.*, 1999). In biological systems, $ONOO^-$ has the potential to nitrate proteins through the formation of nitrotyrosine (Van Der Vliet *et al.*, 1996). Using specific antibodies against the 3-nitrotyrosine residue, protein nitration can be detected by immunological techniques (Ye *et al.*, 1996). The 3-nitrotyrosine formed in proteins *in vivo* has been considered to be 'footprint', or 'marker', for the co-production of NO and O_2^-, followed by $ONOO^-$. However, since peroxidase reactions involving nitrite and hydrogen peroxide (H_2O_2) can also produce nitrotyrosine by a peroxynitrite-independent mechanism, the detection of 3-nitrotyrosine *in vivo* does not definitively prove the formation of NO and O_2^- (Sakihama *et al.*, 2003).

In contrast to vertebrate animal cells, which have fairly constant O_2 levels, O_2 concentrations can vary substantially between different plant tissues. Moreover, the O_2 levels in plant cells are highly dependent on environmental conditions. The green photosynthetic cells of leaves evolve O_2 in the chloroplasts in the light, whereas root cells may be exposed to hypoxic or anoxic conditions in the soils. Furthermore, abiotic and biotic stresses, including pathogen infection, result in enhanced ROS production. Local bursts of O_2 can increase cellular ROS concentrations and thus determine or alter the fate of NO in the cells. Therefore, knowing the *in situ* levels of O_2 and ROS is needed to explore the signalling functions of NO.

5.3 An overview of NO-dependent signalling systems

The NO-dependent signalling pathways in living systems are illustrated in the generalized schematic shown in Figure 5.2. Until recently, only two substrates, nitrite and L-arginine, had been established as the major sources of NO in cells. The nitrite-dependent pathway (nitrite pathway) and the

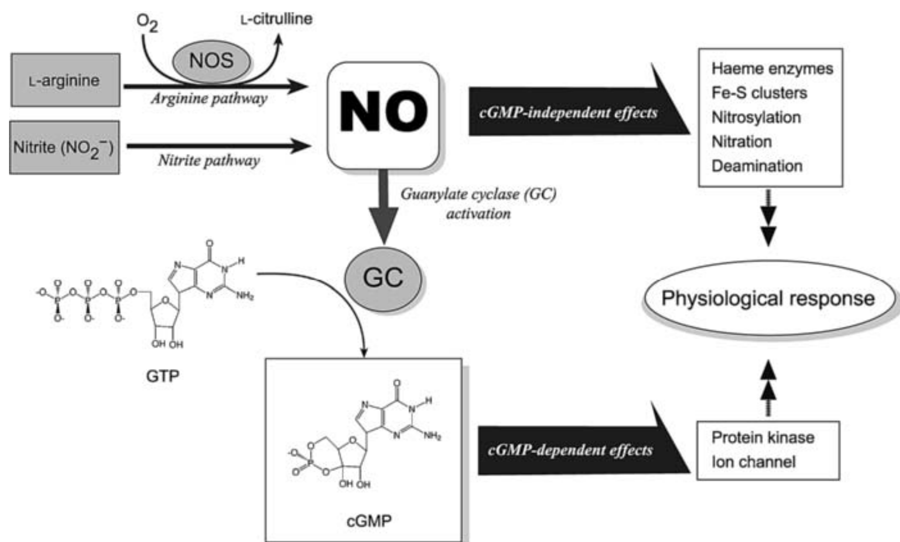


Figure 5.2 Simplified scheme for the NO-dependent signal transduction pathway. For NO production in eukaryotes, L-arginine and nitrite (NO_2^-) have been identified as the substrates (the arginine and nitrite pathways). The formation of NO from L-arginine is mediated by NO synthase (NOS) or NOS-like enzymes. The NO-dependent signal transduction pathway includes cGMP-dependent and cGMP-independent pathways. The second messenger cGMP produced by guanylate cyclase (GC) that is activated by NO is a key step in the cGMP-dependent pathway. cGMP, guanosine 3′,5′-cyclic monophosphate.

L-arginine-dependent pathway (arginine pathway) remain the best described systems of NO production.

The chemical reduction of nitrite produces NO without the aid of enzymes (Yamasaki, 2000). Since the chemical production of NO requires an acidic pH, this reaction may be limited to certain cellular compartments or it may occur only under special conditions (Bethke *et al.*, 2004). Assimilatory nitrate reductase (NR) has been found to produce NO in addition to its nitrate-reducing activity (Yamasaki, 2004). The NO-producing activity of NR was known in legume plants, but this characteristic of the old enzyme had been long overlooked (Yamasaki *et al.*, 1999). Later, nitrite-dependent NO production by NR was confirmed in many plant species (see Chapter 6 for details). It is important to note that NR is not the only enzyme involved in the nitrite pathway (Yamasaki, 2004). There are many potential sources of NO from nitrite in cells (Corpas *et al.*, 2004; Neill *et al.*, 2008), including photosynthetic electron transport in chloroplasts (Jasid *et al.*, 2006) and respiratory electron transport in mitochondria (Gupta *et al.*, 2005; Modolo *et al.*, 2005; Planchet *et al.*, 2005).

The arginine pathway that produces NO from L-arginine was first established in the mammalian system (Ignarro, 2000). Before the enzyme was isolated and purified, the activities were often referred to as guanylate cyclase-activating enzymes (Masters, 2000). NO synthase catalyses the enzymatic

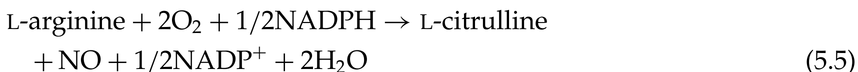
oxidation of L-arginine in the presence O_2 and produces L-citrulline and NO using nicotinamide adenine dinucleotide phosphate (NADPH). NO activates another enzyme, guanylate cyclase (GC), which converts guanosine 5′-triphosphate (GTP) to cyclic GMP (cGMP). The cGMP produced functions as a second messenger, which further activates protein kinases or ion channels. This mechanism is essentially similar in function to cAMP-dependent signal transduction pathways (Ignarro, 2000).

NO also regulates many enzymatic activities without the formation of cGMP. These cGMP-independent effects are ascribed to direct chemical reactions of NO with target biomolecules (Miranda *et al.*, 2000). NO can form a nitrosyl complex with haeme or Fe-S clusters contained in enzymes. Thus, the photosynthetic electron transport chain in chloroplasts and the respiratory chain in mitochondria are inhibited by NO (Yamasaki *et al.*, 2001; Takahashi & Yamasaki, 2002). Moreover, another second messenger in plant signalling cascades, H_2O_2, is produced as a consequence of the inhibition (Yamasaki *et al.*, 2001).

5.4 Mammalian-type NOS – ghost enzymes in plants

Independent investigations concerning NO production and the physiological roles of NO in vertebrates, by Furchgott (1988), Ignarro *et al.* (1987) and the Moncada laboratory (Palmer *et al.*, 1987), revealed that the endothelium-derived relaxing factor was in fact NO. Thereafter, Bredt and Snyder (1990) reported the first isolation and purification of an enzyme that produced NO. This enzyme is now called 'nitric oxide synthase (NOS)'.

All the NOS enzymes found in mammalian systems to date utilize NADPH and O_2 as co-substrates. Three NOS isoforms have been identified in animals. Two of three NOS isoforms are constitutively expressed in cells. The constitutive enzymes are designated as the 'neural isoform (nNOS or type 1)' and the 'endothelial isoform (eNOS or type 3)'. The inducible isoform of NOS (iNOS or type 2) is expressed in many cell types in response to endotoxins and inflammatory cytokines. The different active NOS isoforms are homodimers, each monomer housing a carboxy-terminal reductase domain (NOS reductase domain) resembling cytochrome P450 reductase and an amino-terminal oxygenase domain (NOS oxygenase domain) catalysing cytochrome P450-like chemistry. All NOS isoforms require the following co-factors: flavin adenine dinucleotide (FAD), haeme and tetrahydrobiopterin (BH_4). NOS catalyses the oxidation of L-arginine to L-citrulline in the following reaction:



$$\text{L-arginine} + 2O_2 + 1/2\text{NADPH} \rightarrow \text{L-citrulline} + \text{NO} + 1/2\text{NADP}^+ + 2H_2O \quad (5.5)$$

The first evidence for the presence of NOS activities in plants was reported in 1996 by several different laboratories (Cueto *et al.*, 1996; Leshem & Haramaty, 1996; Ninnemann & Maier, 1996). At about the same time, a number

of physiological functions for NO were proposed in plants; these include the establishment of symbiosis (Cueto *et al.*, 1996), the induction of phytoalexin production (Noritake *et al.*, 1996) and the regulation of plant growth and development (Leshem & Haramaty, 1996). Two landmark papers (Delledonne *et al.*, 1998; Durner *et al.*, 1998) led to the view that plants might possess a mammalian-type of NO signalling system comprising cGMP and NOS. For example, the ability of mammalian NOS inhibitors to block the conversion of L-arginine to L-citrulline suggested the presence of mammalian-type NOS in plants (Cueto *et al.*, 1996; Delledonne *et al.*, 1998). Despite intensive efforts, however, no gene homologues of mammalian NOS have been identified in the genome of *Arabidopsis* (Arabidopsis Genome Initiative, 2000). In contrast, genomic sequence analysis has demonstrated homologues to mammalian NOS genes distributed across the Eukarya domain as well as in the Archaea and Bacteria, which have NOS genes that only contain the canonical oxygenase domain. Although identified in the genome of two species of *Ostreococcus* (Fig. 5.3), the smallest free-living photosynthetic eukaryote (Courties *et al.*, 1994), NOS homologues are conspicuously absent from the genomes of

Figure 5.3 Bootstrap consensus maximum parsimony tree for NOS in eukaryotes and prokaryotes. Bootstrap proportions are shown above internal nodes (nodes on each phylogeny received 100% of the bootstrap replicates). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.

multicellular plants. It is now clear that plants do not possess an NO-producing enzyme that is structurally identical to mammalian NOSs (eNOS, nNOS, iNOS) as previously suggested.

Two types of plant NOS that do not share sequence similarity to mammalian-type were reported in 2003, namely, plant iNOS and AtNOS1. Those plant NOSs were thought to possess unique mechanisms for arginine-dependent NO synthesis and concomitant physiological functions (Guo *et al.*, 2003; Wendehenne *et al.*, 2003). In 2004, two papers describing plant iNOS were retracted (Travis, 2004). In 2006, the NOS activity of AtNOS1 also appeared in doubt (Crawford *et al.*, 2006; Zemojtel *et al.*, 2006). Although AtNOS1 seems to be involved in NO synthesis, the protein does not show any arginine-dependent NO production activity. Therefore, AtNOS1 is not an NOS (Crawford *et al.*, 2006). Accordingly, this protein has been renamed as '*Arabidopsis thaliana* nitric oxide-associated 1 (AtNOA1)'. It was demonstrated that AtNOA1 is a functional GTPase (Moreau *et al.*, 2008) that operates in a G-protein-dependent stomatal closure signalling pathway (Li *et al.*, 2009). The failure to establish either of the two strong candidates, as true enzymes catalysing the arginine-dependent NO production in plants, has left something of a 'gaping hole' in current research in this field. However, an increasing number of reports continue to suggest that an arginine-dependent NO synthesis is present in plants. To date, however, there is no concrete evidence for the presence of a plant protein that exhibits NOS activity.

5.5 Comparative NO-related signalling

As discussed above, previous enthusiastic efforts to discover a mammalian NOS-like enzyme in plants have failed. In the search for a plant NOS, much attention has been focused on possible similarities between molecular conservation mechanisms between the mammalian NOS and the putative plant enzymes. Such similarities have been proposed as the levels of structure (e.g. amino acid sequence homologies with similar immunoreactivities) and function (e.g. substrate specificities and sensitivities to mammalian NOS inhibitors). However, it might now be necessary to expand our field of view, so that instead of considering that putative plant NO-releasing enzymes are the only important factors producing NO in plants, we should also consider the NO signalling systems as a whole including NOS itself and NO-dependent downstream factors. If a mammalian NOS-like enzyme is conserved in plants, it is logical to consider that the corresponding mammalian-like downstream signalling factors might also be conserved.

In animal cells, NO induces the activation of soluble guanylate cyclase (GC), which results in a transient increase of the messenger molecule guanosine 3′,5′-cyclic monophosphate (cGMP) from guanosine 5′-triphosphate (GTP). Then, cGMP transduces the signals by regulating the activities

of downstream components such as cGMP-dependent protein kinases (PKG), cyclic nucleotide-gated ion (particularly Ca^{2+}) channels (CNGC) and phosphodiesterases (PDE). The presence of cGMP has also been demonstrated in plants *Picea abies* (Norway spruce; Pfeiffer *et al.*, 1994), *Hordeum vulgare* (barley; Penson *et al.*, 1996), *Nicotiana tabacum* (tobacco; Durner *et al.*, 1998), *Pharbitis nil* (morning glory; Szmidt-Jaworska *et al.*, 2004, 2008) and *Arabidopsis thaliana* (Donaldson *et al.*, 2004); however, the levels of cGMP are comparatively low. The endogenous cGMP level was reported to rapidly elevate by the external application of gaseous NO or an NO donor on spruce needles (Pfeiffer *et al.*, 1994), tobacco leaves and suspension cells (Durner *et al.*, 1998), *P. nil* cotyledons (Szmidt-Jaworska *et al.*, 2008) and *Glycine max* (soybean) cultured cells (Suita *et al.*, 2009), by gibberellic acid (GA) on barley aleurone layers (Penson *et al.*, 1996), and by salt and osmotic stress on *A. thaliana* seedlings (Donaldson *et al.*, 2004). It is presumed that the NO-responsive elevation of the endogenous cGMP levels is strong evidence for the presence of a mammalian-like NO signalling system in plants. As the next step, it should be examined whether the NO-responsive elevation of the cGMP levels is primarily brought by soluble GC, which is activated by NO, or is due to secondary effects of NO.

The genome sequence of *A. thaliana* (Arabidopsis Genome Initiative, 2000) does not contain canonical sequences for the conserved GC catalytic domain (Schaap, 2005). Nonetheless, Ludidi and Gehring (2003) identified a novel, unusual type of soluble GC from *A. thaliana*, termed AtGC1. AtGC1 is a soluble enzyme that harbours only the final third of the typical GC catalytic domain, and thus the recognized GTP- and Mg^{2+}-binding moieties are lacking from AtGC1. In spite of this, a recombinant glutathione *S*-transferase (GST):AtGC1 fusion protein, purified from *Escherichia coli*, showed Mg^{2+}-dependent GC activity, suggesting that AtGC1 is a functional plant GC that constitutes a new class of GCs. AtGC1-like GC was also found from *Zea mays* (maize; Yuan *et al.*, 2008). The gene for the maize AtGC1 homologue, designated as *ZmGC1*, was shown to be associated with the resistance to the fungus-caused disease 'Gibberella ear rot', indicating a pivotal role of ZmGC1 (and probably AtGC1) in the plant immunity. Importantly, in an *in vitro* experiment using *E. coli* cell extracts, the GC activity of GST:AtGC1 did not depend on the presence or absence of NO (Ludidi & Gehring, 2003). This NO-independent nature of the GC activity in AtGC1 might be attributed to its non-typical architecture. As mentioned above, the typical type of soluble GCs is 200-fold activated by the interaction of NO radicals with its inherent haeme Fe^{2+}. AtGC1, however, does not contain any haeme-binding domains. Taken together, although the mammalian-like NO signalling components, soluble GC and cGMP, are in fact present in plants, the universal existence of the mammalian-like signal transduction pathway downstream of NO is questionable at present. Meanwhile, in soybean, NO-responsive gene expression of flavonoid biosynthetic enzymes was blocked by 6-anilino-5,8-quinolinedione, an inhibitor of soluble

GC (Suita *et al.*, 2009), suggesting that mammalian soluble GC-like enzyme(s) might exist in certain plant species, such as legumes.

The molecular basis for NO-dependent increases in endogenous cGMP levels in plants remains uncertain. One possible explanation is that NO affects plant hormone signalling pathways, particularly GA and abscisic acid (ABA). This interaction could then have an indirect effect on cellular cGMP levels. Utilizing a rationally designed search motif, Kwezi *et al.* (2007) found that the *A. thaliana* brassinosteroid receptor, AtBRI1, contains a GC catalytic core within the cytosolic kinase domain, which is capable of converting GTP to cGMP *in vitro*. Furthermore, this 'extended' search motif for the GC catalytic centre identified 27 putative GC proteins, including AtGC1 and AtBRI1, of *A. thaliana*. Thirteen among the 27 proteins are annotated as leucine-rich repeat receptor-like kinases (LRR-RLKs) including AtBRI1. In this context, it is noteworthy that the completely sequenced unicellular green alga *Chlamydomonas reinhardtii* contains 55 annotated putative GCs (Merchant *et al.*, 2007). These results might imply that plants possess an unexplored wealth of GCs, which would be directly activated by plant hormones and other extracellular ligands, and that cGMP plays a role as a second messenger in phytohormone signalling. The rapid elevation of the cGMP level in *A. thaliana* seedlings by salt and osmotic stress (Donaldson *et al.*, 2004) seems to be caused by hormonal control involving ABA.

In summary, the mammalian-type scheme of an NOS/GC/cGMP signalling pathway seems to be not applicable to plants. The absence of such a pathway raises the question whether a mammalian-like NOS is present in plants. While this has long been believed and eagerly sought, there is no evidence to date of the existence of such a plant enzyme. Further investigations to identify regulatory components upstream of the newly discovered plant GC proteins, including AtGC1 and AtBRI1, would extend our understanding of the conservation of NO signalling systems between mammals and plants.

Similar to plants, no mammalian NOS-like enzymes have been found in fungi and cyanobacteria, while cGMP was detected in the budding yeast *Saccharomyces cerevisiae* (Eckstein, 1988a, 1988b), the bread mould *Neurospora crassa* (Rosenberg & Pall, 1978; Shaw & Harding, 1987) and the cyanobacterium *Synechocystis* sp. PCC 6803 (Cadoret *et al.*, 2005). Although more than a dozen fungal genomes, including those of *S. cerevisiae* and *N. crassa*, have been fully sequenced to date (Espagne *et al.*, 2008), no genes encoding either GCs, PKGs or cGMP-dependent PDEs have been reported (Schaap, 2005). *Synechocystis* possesses a single putative GC, Cya2 (Ochoa de Alda *et al.*, 2000; Rauch *et al.*, 2008). Cya2 is not a soluble enzyme, but an integral membrane protein with four transmembrane helices, which is likely to be located in the plasma membrane. Currently, the responsiveness of Cya2 to NO is unknown. Here again, fungi and cyanobacteria seem to employ NO signalling pathways, which are quite different from the NOS/GC/cGMP pathway of mammals, and hence do not involve a mammalian NOS-like enzyme.

5.6 Algal nitric oxide synthesis – an echo from water

Research on algal NO production is still in its infancy, having only begun when Soeder *et al.* (1996) demonstrated NO production by the green alga *Scenedesmus*. Although there is a general consensus concerning a role for the nitrate reductase (NR) enzyme in the generation of NO in plants (Rockel *et al.*, 2002; Meyer *et al.*, 2005) and some algae (Sakihama *et al.*, 2002), the main source of NO generation in algae remains unclear due to the publication of conflicting data.

In a study performed with the green alga *Scenedesmus* and the cyanobacteria *Anabaena doliolum* and *Synechococcus*, Mallick *et al.* (1999) found that the accumulation of nitrite was responsible for the formation of NO. A subsequent study performed with *Scenedesmus* confirmed that the generation of NO was not L-arginine-dependent in this species and that the common NOS inhibitors, L-N^{G}-nitroarginine methyl ester (L-NAME) and L-N^{G}-nitroarginine (L-NNA), had no effect on the NO generation process (Mallick *et al.*, 2000). In a study performed with the green alga *Chlorella* sp. from Antarctica, the generation of NO occurred concomitantly with the log phase of the algal growth. In this study, both NR- and NOS-like enzyme activities appeared to be contributors to the cellular production of NO (Estevez & Puntarulo, 2005). Unlike in multicellular plants, for which there is yet no evidence of NOS gene homology, the findings of NOS homologues in the complete genome sequences of *Ostreococcus* spp. (Derelle *et al.*, 2006) lend indirect support to the above reports of NOS-like activities in the other green algal species. In addition to the activities of NR and NOS-like enzymes, mitochondrial electron transport has also been implicated in the production of NO from nitrite in a *Chlorella* sp. (Tischner *et al.*, 2004).

Diverse mechanisms of NO production have also been reported in algal species other than the green algae. In the dinoflagellate *Symbiodinium microadriaticum*, a common coral symbiotic microalga, observed nitrite-dependent NO production seemed to result from the NR activity and possibly from mitochondrial enzymes, while the observed L-arginine-dependent NO production was related to an NOS-like activity (Bouchard & Yamasaki, 2008). Other studies performed with coral symbiotic microalgae have also reported the presence of NOS-like activities in heat-stressed *Symbiodinium bermudense* (Trapido-Rosenthal *et al.*, 2001, 2005). Interestingly, in a study performed using the raphidophyte *Chattonella marina*, Kim *et al.* (2006) found increased NO production upon supplementation with L-arginine but no effect upon supplementation with nitrite. In this study, the application of L-NAME, a specific NOS inhibitor, significantly decreased the production of NO, thereby implicating an NOS-like enzyme in the production of NO in this harmful red tide alga. Cells of the marine diatom *Phaeodactylum tricornutum* overexpressing PtNOA displayed higher NO production, suggesting the involvement of the gene in NO signalling mechanisms similar to the case of AtNOA1 in

Arabidopsis. The PtNOA protein is plastid localized (Vardi *et al.*, 2008) as is the plant orthologue (Flores-Perez *et al.*, 2008).

5.7 Nitric oxide synthase in plant-associated bacteria: its occurrence and functions

Energy metabolism by bacteria is responsible for the majority of biologically derived NO, which is typically formed via reduction of nitrite (Jousset *et al.*, 2001; Cohen *et al.*, 2005). Under conditions common in agricultural soils, O_2-limited ammonia-oxidizing nitrifying bacteria will utilize autogenic nitrite as a terminal electron acceptor. Under flooded conditions, such as in rice paddies, denitrifying bacteria are the predominant source of NO (Zumft, 1997), which can be produced in sufficient quantities as to stimulate NO-detoxifying gene expression in neighbouring bacteria (Choi *et al.*, 2006). NO produced by denitrifying *Azospirillum brasilense* bacteria on tomato roots has been shown to activate the NO-responsive root branching pathway (Molina-Favero *et al.*, 2008).

NO can also be produced in some bacteria for autoregulatory or biosynthetic purposes by bacterial NOS (bNOS). Genetic confirmation for the presence of bNOS exists primarily within the Gram-positive bacteria, including many commonly plant-associated species in the orders Actinomycetales (*Mycobacterium*, *Nocardia*, *Rhodococcus*, *Streptomyces*) and Bacillales (*Bacillus*, *Geobacillus*, *Paenibacillus*) (Sudhamsu & Crane, 2009). The catalytic properties of bNOS are similar to the mammalian NOS oxygenase domain, but the enzyme lacks a reductase domain and, therefore, must depend on another protein to provide electrons for O_2 activation. In *Bacillus subtilis*, this physiological role may be served by a variety of reductases; individual reductases that are able to support sustained NO synthesis from bNOS in *in vitro* reconstitution experiments (Wang *et al.*, 2007b) can be deleted without altering bNOS activity *in vivo* (Gusarov *et al.*, 2008).

Endophytic association of actinomycetes and other bNOS-containing bacteria, some of which may be vertically transmitted within seeds, has been demonstrated in diverse plants (Cohen *et al.*, 2006; Rosenblueth & Martinez-Romero, 2006). We believe that, to confidently ascribe the source of identified NOS-like activities in plants, more rigorous demonstration of the axenic nature of assayed plant samples must become standard practice in the field of plant NO biology (Cohen *et al.*, 2006).

Plant-derived disaccharides have been shown to up-regulate NOS activity in two actinomycetes. The phytopathogen *Streptomyces turgidiscabies* induces bNOS activity in response to the cell wall building block cellobiose, and releases levels of NO in colonized growing root zones beyond that needed for nitration of the phytotoxin thaxtomin (Johnson *et al.*, 2008). Many beneficial *Streptomyces* secrete cell wall-degrading enzymes in the process of colonizing

a plant host, thereby releasing cellobiose into their immediate environment (Langlois *et al.*, 2003; Suzuki *et al.*, 2005). It remains to be seen whether NO is produced in the course of these interactions as well. *Rhodococcus* sp. strain APG1, isolated as an endophyte of *Azolla pinnata*, induces bNOS activity in response to sucrose (Cohen & Yamasaki, 2003), which is potentially of physiological significance since sucrose is exuded into the symbiotic leaf cavities of *Azolla* spp. (Peters & Meeks, 1989). Future research should investigate whether host plants respond to bNOS-derived NO.

One clear function for bNOS is in oxidative defence signalling. Microbes in the phyllosphere are faced with a multitude of oxidative stresses, including ROS generated by exposure to UV light (Green & Fluhr, 1995). In *Rhodococcus* strain APG1, bNOS activity is associated with elevated catalase activity and tolerance of the bacterium to H_2O_2 (Cohen & Yamasaki, 2003). In *B. subtilis* and *Bacillus anthracis*, bNOS-derived NO was shown to exert a cytoprotective effect by activating catalase and by inhibiting enzymatic reduction of free cysteine, thereby suppressing production of hydroxyl radicals via the Fenton reaction (Gusarov & Nudler, 2005; Shatalin *et al.*, 2008). The fact that bNOS activity is necessary for engulfed *B. anthracis* cells to survive the oxidative burst in macrophages (Shatalin *et al.*, 2008) may be of relevance to the observation that *B. anthracis* is able to reproduce and exchange DNA in the rhizosphere of plants (Saile & Koehler, 2006) where microbes are exposed to high levels of ROS through the activity of plant oxidases (Cohen *et al.*, 2001) and protozoan feeding (Halablab *et al.*, 1990; Kreuzer *et al.*, 2006). The proportion of *Streptomyces* spp. that display NOS activity was found to be significantly higher in the rhizosphere of apple roots compared to surrounding soil (Cohen *et al.*, 2005). Although our current knowledge is consistent with the idea that bNOS activity confers cytoprotection in the plant host environment, studies to test this hypothesis are needed.

5.8 Prospects for NO-dependent signal transduction systems in plants

Taking all the available information into account, it has become evident that plants do not possess a mammalian type of NOS/GC/cGMP signal transduction system. As a consequence, three scenarios for NO-dependent signal transduction are possible in plants, and these are illustrated in Figure 5.4. Scenario A follows the classical NOS/GC/cGMP pathway, which has been established in mammalian systems. On the source of NO in mammalian systems, recent findings offer a modification of Scenario A. Although eNOS has been presumed to provide NO in the vascular system for vasorelaxation, the enzyme is not responsible for NO production under ischaemia conditions where hypoxia or anoxia occurs (Zweier *et al.*, 1995). NOS requires O_2 to produce NO and thus the enzyme cannot synthesize NO under ischaemic

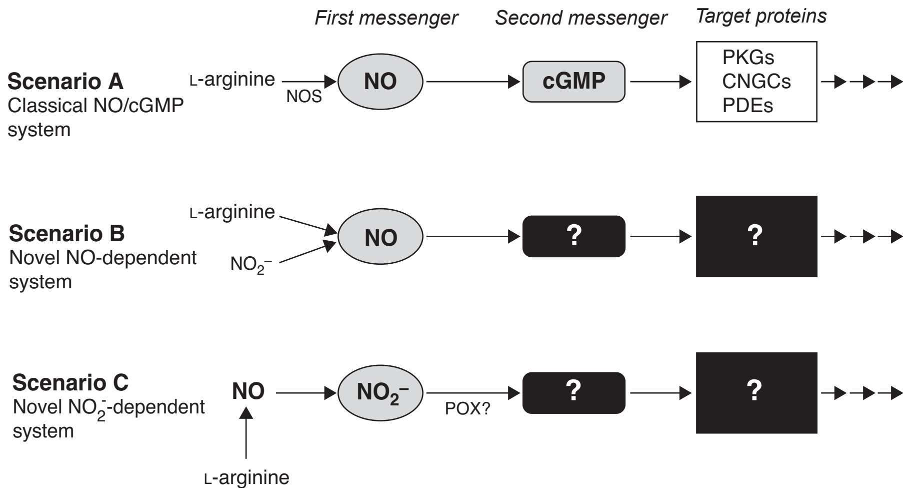


Figure 5.4 Three possible scenarios for NO-dependent signal transduction systems in plants. The square black boxes are hypothetical factors that have not yet been identified. PKGs, cGMP-dependent protein kinases; CNGCs, cyclic nucleotide-gated ion channels; PDEs, phosphodiesterases; POX, peroxidase. See text for details.

conditions (Yamasaki, 2005). Recently, blood haemoglobin has been found to exhibit nitrite reductase (NiR) activity that catalyses NO production using NADH and nitrite (NO_2^-) as the substrates (Dejam *et al.*, 2004; Gladwin *et al.*, 2004), a reaction apparently similar to that of plant NR (Yamasaki *et al.*, 1999; Yamasaki & Sakihama, 2000). Whatever the source of NO (Yamasaki & Cohen, 2006), the activation of GC followed by the formation of cGMP is included in Scenario A (classical NO/cGMP signalling system).

If Scenario A is not applicable to plants, what type of alternative system is plausible? It is our opinion that there is no doubt that plants have NO-producing capabilities. It also appears that GTP may be involved in the plant NO-dependent signal transduction pathways. Assuming that NO acts as a first messenger, we might overlook the necessity for, as yet unknown, second messenger(s), which are derived from NO. The nitration (-NO_2) or nitrosylation (-NO) of biomolecules is mediated through non-enzymatic and enzymatic mechanisms (Neill *et al.*, 2008). We suggest therefore that there is an urgent need to search for such NO derivatives in plants, and explore their roles as possible signalling molecules (Yamasaki, 2004). In 2007, a novel candidate has emerged in this field. Using cultured macrophage as a model, Sawa *et al.* (2007) have demonstrated unique chemical and biological properties of 8-NO_2-cGMP that are entirely different from the known NO/cGMP signalling system. The discovery of a novel second messenger derived from NO now leads us to set Scenario B (novel NO-dependent system).

NO produces NO_2^- as an oxidation product. The product NO_2^- is a substrate for NO production. This circularity of the reactions may offer a

paradoxical hypothesis; NO_2^- (but not NO) would be the first messenger (Scenario C, novel NO_2^--dependent system). In fact, recent analysis has shown that NO_2^- increases mRNA levels of several nitrate-inducible genes as rapid as nitrate in nitrogen-starved *Arabidopsis* roots (Wang *et al.*, 2007a). Haemeperoxidases, including horseradish peroxidase, myeloperoxidase and lactoperoxidase, are capable of nitrating tyrosine residues through nitrogen dioxide radical (NO_2) formation (Sakihama *et al.*, 2003). Interestingly, plant haemoglobins (GLB1, GLB2, GLB3) possess peroxidase activity, and they can nitrate themselves in the presence of NO_2^- and H_2O_2 (Sakamoto *et al.*, 2004). The requirement of co-factor(s) such as H_2O_2 might account for cross-talk between RNS and ROS in signal transduction systems in plants. Those scenarios described above, of course, need to be tested and await breakthrough results in this field.

5.9 Concluding remarks

Since the last decade, plant scientists have been repeatedly surprised and sometimes confused by the discoveries in the NO field of research. Much confusion has arisen because there has always been a strong presumption that plants possess an NO-dependent signalling system analogous to mammalian pathways. However, it should be remembered that plants and animals have entirely different relationships with the environment. As plants cannot move to avoid stressful environmental conditions, their growth and development is more closely influenced by environmental triggers and, endogenous NO levels vary according to presence of biotic and abiotic cues (Fig. 5.1). Roots are often exposed to a dynamic soil environment where NO levels can drastically change as a result of bacterial activities. NO is abundant in the environment, it reacts with many types of molecules and it is membrane-permeable. These features mean that plant NO signalling systems are directly exposed to NO, without the need for the complex nerve transduction system present in animals. Since NO donors can induce many physiological responses (Neill *et al.*, 2008), plant cells appear to be open for exogenous NO. In contrast, animal cells in tissues (of vertebrates in particular) are shielded by abundant haeme proteins, including haemoglobins and myoglobins, that bind or degrade NO. These fundamental differences may have led to the evolution of distinctive mechanisms for signal reception (Yamasaki, 2005).

Other important differences may be present in the unique features of metabolism in plants and animals. Photosynthesis, nitrate assimilation and sulphate assimilation take place in the green cells of plants. These assimilation pathways produce large amounts of reactive by-products, such as O_2 and ROS from photosynthesis (Yamasaki, 2000), NO and RNS from nitrate assimilation (Yamasaki, 2004), and hydrogen sulphide (H_2S) from sulphur assimilation (Rausch and Wachter, 2005). These reactive by-products often interact with each other at high rates, and they show similar affinities for

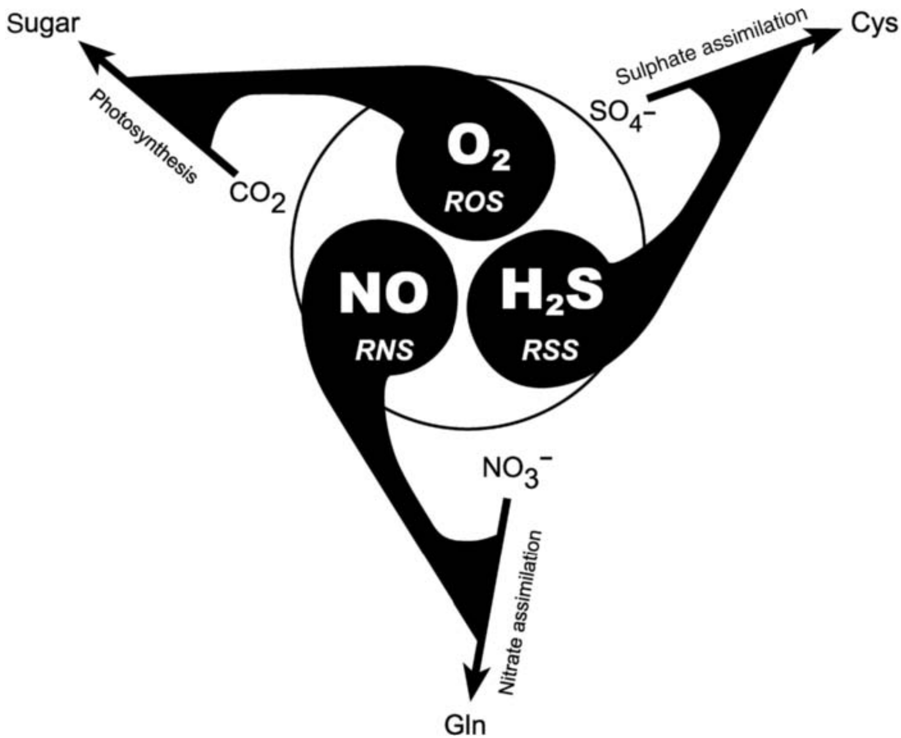


Figure 5.5 Conceptual diagram for a harmonic interplay of photosynthesis, nitrate assimilation and sulphate assimilation. Three assimilation processes may be orchestrated by reactive by-products that are potentially produced during the metabolisms (Yamasaki, 2005). ROS, reactive oxygen species; RNS, reactive nitrogen species; RSS, reactive sulphur species. See text for details.

haeme proteins (Yamasaki, 2005). Interplay between these reactive molecules could be a key for future studies, not only concerning the NO-dependent signalling systems in plants, but also the network of interfacing regulatory mechanisms that exist between the different assimilation processes (Fig. 5.5).

Often the major target molecules, such as proteins, sugars, lipids, DNA or RNA that are intensively studied in the plant field using biochemistry and molecular biology, tend to be stable, large molecules that are unique in structure. The fundamental nature of NO is rather different to such conventional biomolecules in that NO is unstable, small, simple and ubiquitous. Thus, conventional molecular genetic or physiological approaches are not always applicable to the investigation of NO signalling or the analysis of the biological functions of NO. While it is essential to establish a more straightforward direction for NO research in the post-genomic era, it is becoming increasingly clear that a paradigm shift is needed to achieve a complete understanding of the biological functions of NO in plants.

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Chapter 6

NITRATE REDUCTASE AND NITRIC OXIDE

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Abstract: Plants produce NO either by reduction of nitrite or by oxidation of more reduced N forms. Reductive NO formation from nitrite can be enzymatic via nitrate reductase (NR), mitochondrial electron transport or non-enzymatic at acidic pH. In any case, NR is the major (if not the only) source for endogenous nitrite in plants. Therefore, NR properties and regulation are summarized here with respect to NO production. Reductive NO formation from L-arginine or from hydroxylamines is also considered. Problems in quantifying NO production from nitrite and potential roles of NO in plants are discussed in context with nitrate reduction.

Keywords: nitrate reductase; nitric oxide; nitrite; NO signalling; NR regulation

6.1 Introduction

In plants, like in mammals, nitric oxide (NO) appears to play numerous and important roles as a short-lived signalling molecule. It is, therefore, astonishing that exact knowledge on various sources for NO is still one of the most confounding issues in plant research. The following reactions for nitric oxide production in plants are under debate:

Reductive NO formation from nitrite

(a) Nitrate reductase (NR, cytosolic or plasma membrane-bound)
(b) Xanthine dehydrogenase

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(c) Mitochondrial cytochrome *c* oxidase
(d) Non-enzymatic by acid pH

Oxidative NO formation from L*-arginine or hydroxylamines*

(e) Nitric oxide synthase (NOS)
(f) Hydroxylamine-oxidation by reactive oxygen (ROS)

Reductive NO formation depends exclusively on nitrite as substrate, which can be produced within the plant only by nitrate reductase (NR, E.C. 1.6.6.1.). In root systems, additional nitrite may be taken up from the soil, where it may be formed during microbial nitrification or denitrification.

Reaction (e) uses L-arginine, nicotinamide adenine dinucleotide phosphate (NADPH) and O_2 as substrates. In mammals, the reaction is catalysed by a family of NO synthases (NOS, E.C. 1.14.13.39). In 2003, two types of NOS (iNOS in tobacco and AtNOS1 in *Arabidopsis*) were also reported to exist in plants, but both had little sequence similarity to the mammalian-type NOS. The data on iNOS were later retracted, and AtNOS1 was shown to be no NOS, although somehow involved in NO formation (Crawford *et al.*, 2006; Zemojtel *et al.*, 2006; Tischner *et al.*, 2007).

Recently, a second type of oxidative NO formation is detected: plant cells are able to oxidize added hydroxylamines to NO (Rümer *et al.*, 2009). Reaction (f) works already at low micromolar substrate concentrations and, like NOS, requires oxygen, and ROS appeared involved as oxidants. Due to the uncertainties about the existence and concentrations of natural hydroxylamines in plants, the physiological relevance of that reaction is presently unclear.

Thus, at this point, nitrite-dependent NO formation is the only NO source in plants which is well established and quantified, and for which genes and enzymes and their transcriptional and post-translational regulation have been intensively investigated. As mentioned, nitrite can be converted to NO by NR itself, or by mitochondrial electron transport (c) or by acidic conversion (d), whereas a role for xanthine dehydrogenase (b) also appears doubtful. Considering the fact that NR is the only source for nitrite at least in plant leaves, the enzyme should be of extraordinary importance, not only as a major catalyst in the N-assimilation pathway, but also as a source for NO in development, stress response and pathogen defence. Therefore, it seems justified to briefly summarize knowledge on NR in general, and specifically on NO formation by NR.

6.2 Structure, basic functions and regulation of NR

6.2.1 Structure and basic function

Numerous articles have described structural and functional aspects of NR, and we summarize present knowledge only briefly (Fig. 6.1). Plant NR

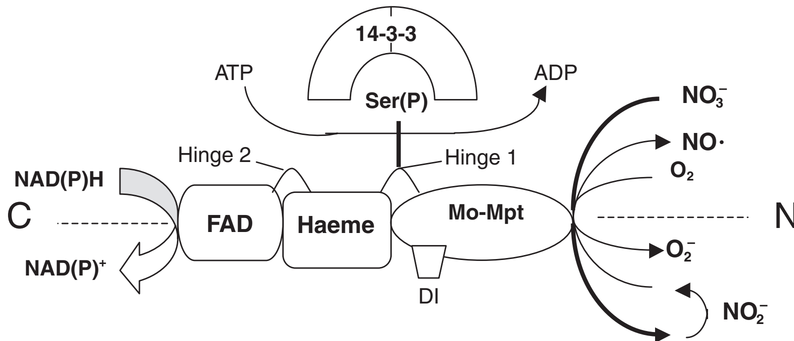

Figure 6.1 Structural and functional scheme for NR. The FAD domain accepts two electrons from NAD(P)H. The electrons are transferred via haeme-Fe to Mo-MPT, where nitrate (+5) is normally reduced to nitrite (+3) in a two-electron transfer reaction. Alternatively, two different one-electron transfers may take place at the Mo-MPT domain, either to molecular oxygen to produce the superoxide anion, or to nitrite to produce the free radical $NO^{\cdot}$. The one-electron transfer reactions occur probably both with only a small part (1%) of the normal nitrate-reducing capacity (Rockel *et al.*, 2002). Hinge 1 carries the regulatory serine residue (position 534 in *Arabidopsis* and 543 in spinach), which upon phosphorylation becomes part of a 14-3-3-binding motif. In the presence of divalent cations, a 14-3-3 dimer (not shown) will bind to that motif and change the structurally flexible hinge 1 apparently in such a way that the electron transfer from haeme to Mo-MPT is interrupted, thus rendering NR inactive. 'DI' marks the dimerization interface domain. For further details, see text and Campbell (2002).

normally catalyses the NAD(P)H-dependent two-electron reduction of nitrate (+5) to nitrite (+3). The soluble enzyme is located mainly in the cytosol, but existence of some plasma membrane-bound analogue has also been reported (Stöhr & Ullrich, 1997). The monomeric subunit of cytosolic NR has two active sites, one for NAD(P)H to donate electrons and another for reduction of nitrate to nitrite. The 100 kD polypeptide with about 900 amino acids contains the co-factors flavin adenine dinucleotide (FAD) (C-terminal), haeme-Fe and molybdenum-molybdopterin (Mo-MPT), bound into structurally independent domains. Two structurally flexible 'hinge' regions on both sides of the haeme domain are connecting it with the Mo-MPT (hinge 1) and with the FAD domain (hinge 2). The dimer interface is located between hinge 1 and Mo-MPT (for review, see Campbell, 2002; Fig. 6.1).

6.2.2 Specific one-electron transfer reactions

In addition to its basic role in catalysing a two-electron transfer from NAD(P)H to nitrate, NR has been shown to also catalyse one-electron transfer reactions. NR can use molecular oxygen as an electron acceptor to produce the superoxide anion (Ruoff & Lillo, 1990), primarily at the Mo-MPT centre (Barber & Kay, 1996; cf. Fig. 6.1). Similarly, nitrite can be reduced to NO, and as the reaction is competitively inhibited by nitrate (Rockel *et al.*, 2002), it probably also occurs at the nitrate reduction site of the Mo-MPT centre. Both

reactions may proceed simultaneously in NR solutions, and the two products, NO and superoxide, may interact to form the highly reactive peroxynitrite (Yamasaki & Sakihama, 2000).

6.2.3 NR regulation

Cellular nitrate concentrations are major factors controlling NR transcription (and transcription of many other N-metabolizing genes). The other important factors inducing NR are sugar levels and/or photosynthesis. Light induction of NR is mediated not only by photosynthesis via sugar levels but also by phytochrome. In the *Arabidopsis* phytochrome-deficient double mutant *hy5 hyh*, the diurnal modulation of NR levels was abolished and NR levels remained very low (Jonassen *et al.*, 2008). The sugar and light response of NR expression together ensure that the enzyme is only synthesized if substrate (NO_3^-) and carbon skeletons for amino acid synthesis are available. Superimposed on transcriptional regulation is the post-translational control of NR by reversible phosphorylation of a Ser residue in the hinge 1 region, for example at position 534 in *Arabidopsis* and 543 in spinach (Bachmann *et al.*, 1996a, 1996b) and 521 in tobacco. Participating protein kinases and phosphatases have been partly identified and described. Phosphorylation as such does not change NR activity, but binding of a 14-3-3 dimer to the phosphorylation motif in the presence of Mg^{2+} renders NR inactive. In leaves, light or photosynthesis appears as a major trigger activating NR by Ser dephosphorylation (for review, see Campbell, 2002).

6.3 NR-dependent NO formation *in vivo*, measured as NO emission

Many methods exist to measure NO concentrations in cells and tissues, or to measure NO production. All of them have distinct advantages and disadvantages. A very sensitive and specific, yet non-invasive, method is gas-phase chemiluminescence detection, which has been used to follow NO production by intact plants, leaves, roots, cell suspensions or enzyme solutions. Much of our work on NR and NO has been carried out with this method, and therefore results obtained with gas-phase chemiluminescence will occupy a major part of this chapter. This does not mean that other methods are bad and should be neglected – it just reflects our own methodical possibilities and preferences.

6.3.1 NO emission from leaves

The early work by Klepper (1978, 1979, 1987) and Harper (1981) has provided the first evidence that legume leaves emit NO into the gas phase and that a constitutive NR is involved in NO emission. Klepper (1990) was also the

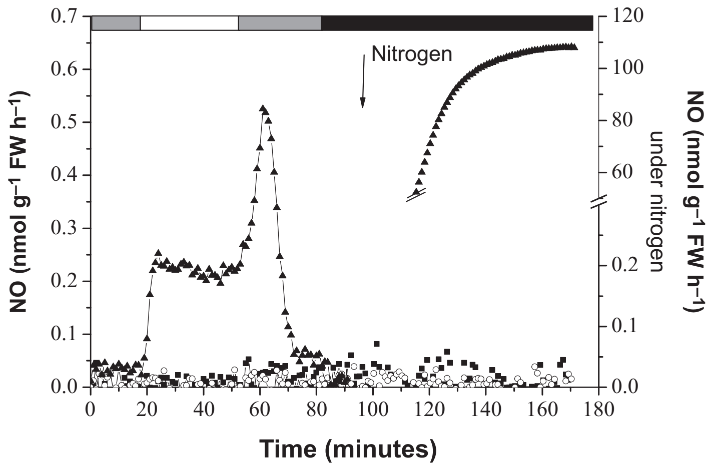

Figure 6.2 NO emission from detached tobacco leaves (*Nicotiana tabacum* cv. Xanthi) as measured by gas-phase chemiluminescence. Each datum point was obtained when the analyser sampled gas during a 20-second period. Thus, the curves are rate curves. The triangles (-▲-) are from normal WT leaves well fertilized with nitrate. NO emission is extremely low in the dark in air. It increases about five-fold in the light. Upon light off, a typical transient peak of NO emission takes place, which is due to a transient nitrite accumulation. The latter is because the dark inactivation of NR is much slower than the almost immediate stop of nitrite reduction upon darkness. Under anoxia (nitrogen, indicated by an arrow) in the dark, the NO emission rate increases more than 200-fold over air. At the same time, nitrite accumulates in the leaf to mM concentrations. The two lower curves represent data from leaves of an NR-free *nia* double mutant (-■-), or from WT plants cultivated on ammonium as the sole nitrogen source (-○-). Under these conditions, NR is not induced. Note that even in nitrogen, when NO emission from WT leaves is very high, nia leaves or 'ammonium leaves' emit almost no NO. Thus, here NR seems to be the major, if not the only, NO source. Data are from Planchet *et al*. (2005).

first to show absence of NO emission from a soybean mutant deficient in a constitutive NR (cNR) present in legumes. More work using *nia* mutants of *Chlamydomonas* (Sakihama *et al*., 2002), *Arabidopsis* (Desikan *et al*., 2002; Magalhaes *et al*., 2002; Modolo *et al*., 2005) and tobacco (Planchet *et al*., 2005), containing no measurable NR activity, confirmed with different NO detection methods that in the absence of inducible NR, NO is not produced, and thus, NR appears to be the dominant NO source in plants (cf. Fig. 6.2).

As mentioned above, full NR expression requires nitrate. When plants are grown on ammonium, instead of nitrate, they develop well, yet their extractable NR activity in leaves is usually below the detection limit. Leaves from such ammonium-grown plants also emit no NO into the gas phase (Planchet *et al*., 2005; Fig. 6.2).

Altogether, it is now beyond any doubt that NO emission from leaves into purified air requires an active NR. Reactions (a–d) listed above all depend on nitrite as the substrate, and because NR is the only source of nitrite

in leaves, all nitrite-dependent NO production requires NR, eventually with the exception of roots in nitrite-rich (e.g. hypoxic) soils. Only reactions (e) and (f) are nitrite-independent. Thus, absence of NO emission from NR-deficient healthy leaves indicates that the contribution of a putative (constitutive) NOS or of hydroxylamine oxidation to total NO emission of a leaf is negligible, at least under normal conditions. Importantly, ammonium-grown plants are often characterized by high levels of L-arginine, and therefore a putative NOS should not be under L-arginine limitation. Nevertheless, existence of an inducible NOS-like activity in plants cannot be excluded and is still under debate (for a recent review, cf. Neill *et al.*, 2008). The above-mentioned experiments with NR-deficient mutants simply indicate that such a potential NOS activity or hydroxylamine-dependent NO formation is low compared to NR-derived NO production.

6.3.2 Light/dark modulation of NO emission *in vivo*

Typically, NO emission from intact photosynthesizing leaves, as measured by gas-phase chemiluminescence, follows a complex time course: emission is low in the dark and higher in the light, sharply contrasting previous measurements by Klepper (1990). Upon a sudden transition from light to dark, a rapid and transient burst of NO takes place (cf. Figs 6.2 and 6.3; Rockel *et al.*, 2002), which is mainly due to a transient overshoot of nitrite concentrations.

6.3.3 Stimulation of NO emission by anoxia

NO emission from NR-containing wild-type (WT) leaves in the dark was strongly elevated under anoxia in the dark (Fig. 6.2). Under these conditions, the *nia* double mutant produced no NO, indicating that NR was the NO source. However, this is to be expected since both reactions (e) and (f) leading to oxidative NO formation (see above) require oxygen. Strong anoxic stimulation of NO emission also took place in root segments or in tobacco cell suspension cultures (Gupta *et al.*, 2005; Planchet *et al.*, 2005). Thus, one interesting question is – Why is NR-dependent NO emission so high under anoxia, and so low in air? There are two possibilities: (i) NO production in nitrogen is much higher than in air, or (ii) NO production is the same in air or in nitrogen, but in air much of the NO is scavenged, for example by rapid oxidation, and thereby escapes detection. As shown in Figure 6.4, NO emission from solution of purified NR is almost the same in air and in nitrogen, whereas in a more complex crude leaf extract, NO emission in nitrogen was about 12 times higher than in air, which might indicate that, in a complex cellular environment, NO scavenging and oxidation is one important factor leading to low aerobic NO emission rates. Another factor probably lies in anoxic nitrite accumulation in plant tissues. NR-expressing plant tissues or cells accumulate nitrite under anoxia to millimolar concentrations, thus creating a very high substrate concentration for NO production. There are several

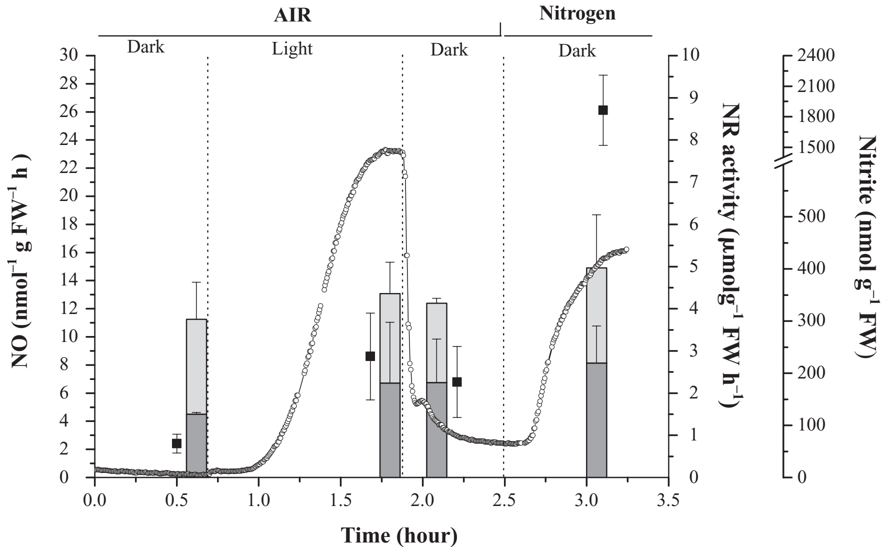

Figure 6.3 NO emission from leaves of an NiR-deficient tobacco mutant with normal NR activity. Leaves were harvested at the end of the dark phase. Initial NO emission in the dark was only 3–4 times higher than in normal WT leaves (cf. Fig. 6.2). In the light, NO emission increased dramatically, reaching 100 times the rate of WT. The dark squares indicate the nitrite content of the leaves, which is about 10- to 20-fold higher than in WT. Notably, after illumination, NO emission drops sharply in the dark, although nitrite concentrations in the leaf have not changed much and are exceeding the K_m nitrite of NR two- to three-fold. Under nitrogen in the dark, emission from the mutant increased again, reaching an about five-fold higher value than in the preceding dark period, almost in parallel with the nitrite concentration, which increased six-fold. Note that upon a similar transition from air (dark) to nitrogen, NO emission from WT leaves increased almost 1000-fold (cf. Fig. 6.2). The columns give NR activity in leaf extracts. Dark grey is the activity + Mg^{2+}, and total column height is the activity in EDTA. The difference between both (light grey area) represents the relative amount of phosphorylated and inactivated NR. Under the above conditions, the light/dark modulation of NR was obviously not well expressed. Data are from Planchet *et al*. (2005).

reasons for that: in all plant tissues, NR becomes activated under anoxia. This activation can be mimicked by treating leaf tissues with permeating weak acids at low external pH (Kaiser & Brendle-Behnisch, 1995), and is therefore thought to be mediated by cytosolic acidification. The exact mechanism for anoxic NR activation (dephosphorylation) is not yet known. Eventually, the protein kinases for NR phosphorylation are more pH-sensitive than the phosphoprotein phosphatases, and not working in an acidified cytosol. In leaves, reduction of nitrite to ammonia via the chloroplast electron transport chain and ferredoxin (Fd)-nitrite reductase is very low in the dark. In consequence, nitrite accumulates when NR is activated. In non-green tissues, nitrite reduction is light-independent and is strongly impaired under anoxia, probably because the oxidative pentose phosphate pathway, which provides reductand for plastidic nitrite reduction, is negatively affected by cellular

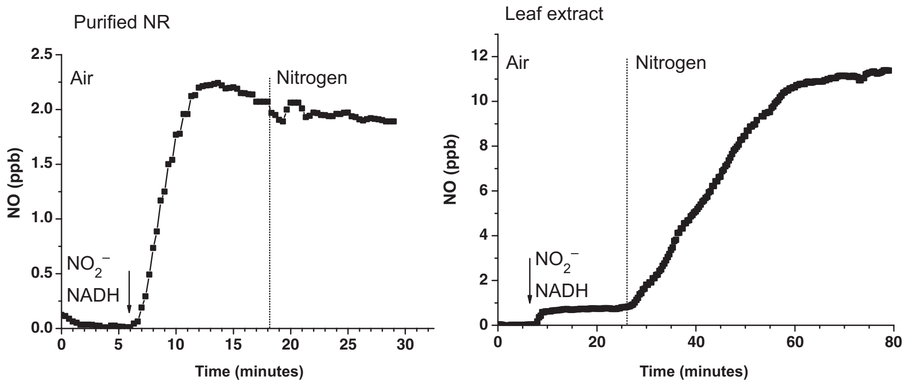

Figure 6.4 Response to oxygen (air) of NO emission by purified NR and by a desalted leaf extract. NO emission by purified NR is the same in nitrogen as in air, indicating that oxidative NO scavenging is irrelevant in that system. In contrast, a leaf extract behaves to some extent like an intact leaf, giving much lower NO emission in air than in nitrogen, in spite of equal substrate concentrations. Apparently, a leaf extract contains compounds that mediate oxidative NO scavenging in air. Normal NR activity (nitrate to nitrite reduction) was not at all affected by air or nitrogen (not shown). For purified NR, data are similar as in Planchet *et al*. (2005). The experiment with crude leaf extracts is unpublished.

acidification (Botrel *et al.*, 1996). Here again, NR activation and impaired nitrite reduction led to nitrite accumulation for high anoxic NO production.

The important role of the cytosolic nitrite concentration as a determinant of NO production rates is impressively demonstrated using leaves of the nitrite reductase (NiR)-deficient transformant tobacco line 271 (Morot-Gaudry-Talarmain *et al.*, 2002; Planchet *et al.*, 2005; Fig. 6.3). When the transformants were grown on nitrate plus ammonium, they had normal or even somewhat elevated NR expression and activity, yet very little NiR activity. In consequence, nitrate-fed plants accumulated nitrite in their leaves to millimolar concentrations. Their NO emission in air in the light was more than 100-fold higher than that of the WT (cf. Figs 6.1 and 6.3). Unexpectedly, rates were similar in light–air and in nitrogen–dark. Whole mutant leaf nitrite levels were always significantly above the K_m of NR for nitrite (100 μM; Rockel *et al.*, 2002) and should therefore have little effect on the rate of NO formation. Nevertheless, NO emission rates were still strongly modulated by light or by the presence and absence of CO_2 (Fig. 6.3). Obviously, in these nitrite-accumulating mutants, NO emission was largely governed by the modulation of NR, with only little limitation by nitrite. Remarkably, the strong modulation of NO emission was only weakly reflected by the NR activation state measured in leaf extracts (Planchet *et al.*, 2005). This may indicate that the standard method for the determination of the NR activation state (comparison of NR activity in extracts with ethylenediaminetetraacetic acid (EDTA) or with Mg^{2+}) may underestimate the real change in NR activity *in vivo*.

Alternatively, or in addition, cytosolic NAD(P)H may also become limiting under aerobic conditions (Kaiser *et al.*, 2002), but direct measurements of cytosolic NAD(P)H are rare.

6.4 NO production by NR *in vitro*

In spinach leaf extracts desalted twice, NO was almost immediately emitted from a stirred solution after addition of substrates, nitrite plus NADH (nicotinamide adenine dinucleotide plus hydrogen) (cf. Planchet *et al.*, 2005). Similar kinetics were reported using NO electrodes (Yamasaki & Sakihama, 2000). In contrast, when nitrate was used as a substrate, NO emission developed slowly, reflecting the requirement for nitrite accumulation in the reaction medium (not shown). Like *in vivo*, NO emission from nitrite plus NADH by leaf extracts was much higher in nitrogen than in air (Fig. 6.4). A preparation of purified NR from maize also produced NO after substrate addition. However, in that simple system, emission rates were almost identical in air and in nitrogen (Fig. 6.4). An explanation would be that crude, desalted extracts contain high-molecular-weight components that partly scavenge NO or that catalyse NO oxidation. In the highly purified NR preparation, these scavenging components are obviously lacking.

As mentioned above, nitrate acts as a competitive inhibitor of nitrite-dependent NO production by NR (Rockel *et al.*, 2002), suggesting that the one-electron reduction of nitrite to NO occurs at the Mo-MPT centre. This suggestion was confirmed by the observation that growth of plants with tungstate, instead of molybdate, for some time before NR extraction prevented NO formation (Planchet *et al.*, 2005).

Expectedly, post-translational modulation of NR by reversible Ser phosphorylation and 14-3-3 binding also modulates NO production. *In vivo*, feeding the cell permeant 5′-AMP (5′-adenosine monophosphate) analogue aminoimidazole carboxamide ribonucleotide (AICAR) through the leaf petiole activated NR, and it also increased NO emission from leaves in the dark. Likewise, modulation of NR *in vitro* by treatment with ATP plus a protein phosphatase inhibitor, which inactivates NR, completely suppressed NO production from nitrite plus NADH (Rockel *et al.*, 2002).

Further evidence for control of NO production through post-translational NR modulation came from genetically modified tobacco, where the regulatory Ser had been replaced by Asp. The modification completely changed the above-described light/dark pattern of NO emission, resulting in higher NO emission in the dark than in the light (Lea *et al.*, 2004). The explanation of that reversed pattern is that genetically modified NR did not bind 14-3-3's any more and was always active. NO emission was lower in the light than in the dark, because normal light-dependent nitrite reduction decreased nitrite concentrations, not only within the plastids, but also in the cytosol. In contrast, in WT leaves nitrite concentrations were always higher in the light

than in the dark (Rockel *et al.*, 2002). In that context, it is important to be aware that mitochondria in leaves are hardly able to reduce nitrite to NO, quite in contrast to mitochondria from non-green plant tissues (Gupta *et al.*, 2005; Planchet *et al.*, 2005).

In experiments with crude leaf extracts and also with purified NR, the maximum NO production rates in nitrogen were only 1–2% of the normal nitrate-reducing activity. Remarkably, rates of oxygen reduction to superoxide by NR were at maximum 0.5% of the activity using nitrate as the terminal acceptor (Barber & Kay, 1996). The similarity of the maximum relative rates of both one-electron transfer reactions suggests that the limitation is inherent for the one-electron transfer itself. In roots or suspension cells, where mitochondria participate in nitrite to NO reduction, oxygen may also have a direct effect on NO production by competitively inhibiting electron transport at CytOx (Gupta *et al.*, 2005).

In summary, although electron flow to nitrite is only a small percentage (~1%) of normal electron flow through NR, all factors known to modulate NR transcriptionally and post-translationally appear to modulate NO production as well. The same probably holds for oxygen reduction through NR, but this has not yet been investigated. Like nitrite production, nitrite consumption by NiR is variable, and any imbalance between nitrate and nitrite reduction will change nitrite levels in cells, thereby contributing to the modulation of NO production.

6.5 Physiological effects of NR-derived NO

The high reactivity of the free radical NO suggests reaction with many potential cellular targets and via different reaction mechanisms, for example *S*-nitrosylation, nitration of aromatic groups or reversible formation of nitrosyl-haeme (summarized in Fig. 6.5). Without presenting details on possible reaction mechanisms and without summarizing the plethora of reports on NO signalling in general, only a few examples are discussed where NR-dependent NO has been considered as 'signalling NO'. More details on the action of NO in plants are given in other chapters.

6.5.1 Effects of NO on NR itself?

As mentioned, NO may react with haeme groups, by forming NO-Fe adducts. For example, NO has been reported to inhibit CytOx in the mitochondrial electron transport chain, with a $K_{0.5}$ of 0.1–0.3 μM (Caro & Puntarulo, 1999; Yamasaki *et al.*, 2001; Millar *et al.*, 2002). Whether such NO concentrations in the low micromolar range are really reached *in situ* is still uncertain. But as NO interacts with haeme, it seems possible that it would also affect NR itself, as suggested by Garcia-Mata and Lamattina (2003). Some direct evidence was provided by Du *et al.* (2008), who reported NO to activate NR

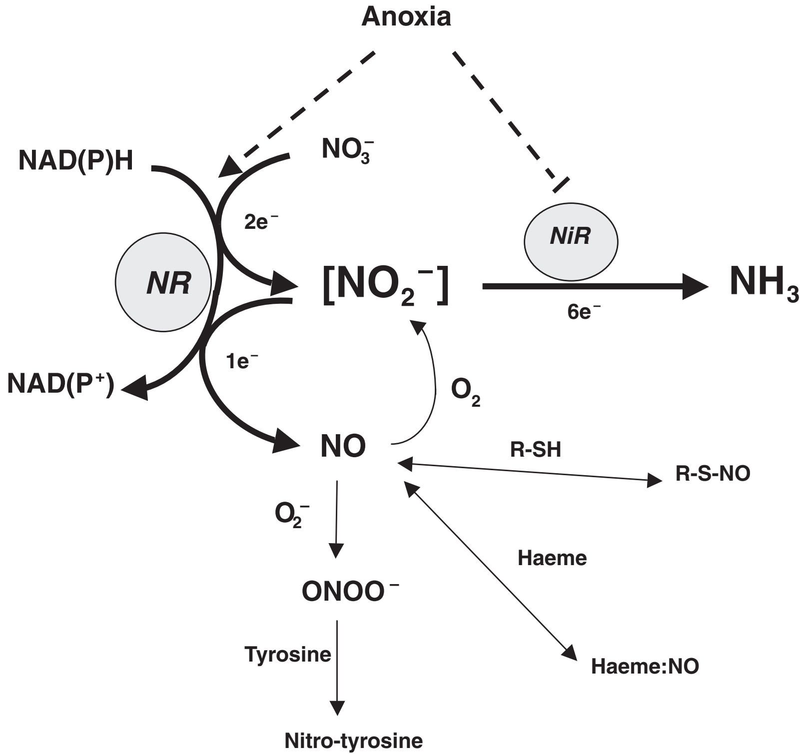

Figure 6.5 Reactions affecting the nitrite concentration and the fate of NO. The nitrite concentration depends on the relative rates of nitrate reduction and nitrite reduction to ammonium. The most striking example for a disturbance of the equilibrium between nitrate and nitrite reduction is observed under anoxia, where NR is fully activated, yet nitrite reduction is strongly impaired. This leads to nitrite accumulation and subsequently to very high NO emission. High NO emission is favoured additionally, because autoxidation of NO to nitrite or reaction of NO with superoxide radicals is absent. NO may be also scavenged by a number of other reactions, including formation of haeme adducts, of nitrosothiols, or nitration (via peroxynitrite) of tyrosine (also compare the review by Neill *et al*., 2008).

by interacting with both the haeme and the MoCo domain. NR activity was decreased by low concentrations of the NO scavenger 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), and was increased by the NO donor sodium nitroprusside (SNP) in the assay solution (Du *et al*., 2008). However, these results require further investigation for several reasons. First, it is difficult to understand how the reaction of NO with haeme should in one case (mitochondrial electron transport chain) inhibit electron flow, and in the other case (NR) activate or facilitate electron flow through haeme respectively in cytochrome. Second, contrary to Du *et al*. (2008), we observed that purified NR from maize was not stimulated, but strongly

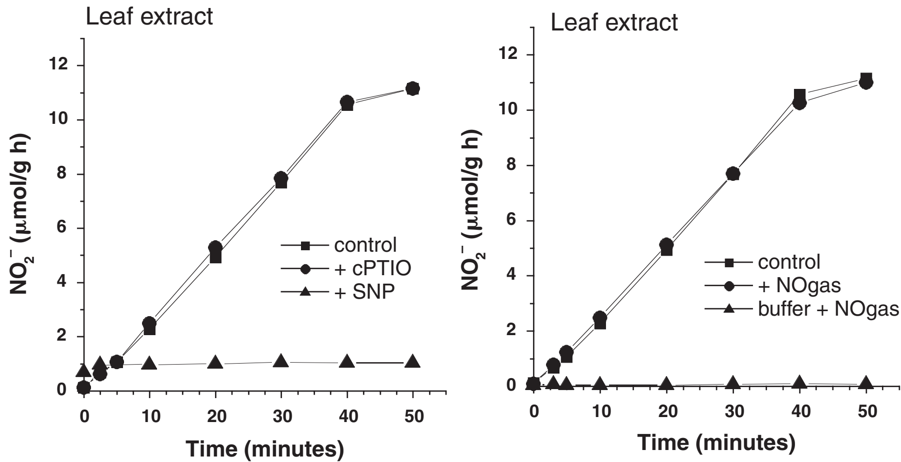

Figure 6.6 Are there direct effects of NO on NR? A commercial preparation of purified maize NR was assayed for the indicated times in the absence (control) or presence of the NO scavenger cPTIO (20 μM) or the NO donor SNP (20 μM), or with a freshly prepared NO solution, obtained by pre-flushing the complete reaction buffer without NR with NO gas (500 ppb in nitrogen) for 10 minutes before addition of NR. The final concentration of dissolved NO was 0.95 nM. As a further control, aliquots of the reaction buffer were pre-flushed with NO but without NR were sampled at the indicated times to check for nitrite accumulation due to direct NO oxidation (buffer + NO gas). The figure on the left shows that the NO scavenger cPTIO had no effect on NR, whereas the NO donor SNP caused an almost complete inhibition, which is probably due to production of HCN by SNP. The figure on the right shows that in a buffer solution (-△-), nitrite formation by NO autoxidation was negligible. It also shows that NO gas had no effect on NR activity (-◇-, unpublished data). For further explanations see text.

inhibited by low concentrations of the NO donor SNP (Fig. 6.6). This is not unexpected, as SNP is known to produce not only NO but also hydrogen cyanide (HCN) (Bethke *et al.*, 2006a), which inhibits NR. Addition of a solution of NO (prepared by flushing the NR reaction buffer for 10 minutes with 500 ppb NO in nitrogen) to the NR reaction medium had no effect on NR activity (not shown). The NO scavenger cPTIO (2 and 20 μM) in our hands had no effect at all on NR when directly added to the NR assay solution (Fig. 6.6), again contrary to the report by Du *et al.* (2008).

6.5.2 NO in stomatal movement

NO donors decrease transpiration in water-stressed wheat and close stomata of epidermal strips of *Tradescantia* and Faba bean (Garcia-Mata & Lamattina, 2001). NO was also shown to be required for abscisic acid (ABA)- or CO_2-induced stomatal closure, and ABA as well as high CO_2 enhanced NO synthesis in guard cells (Neill *et al.*, 2002; Kolla & Raghavendra, 2007), as detected with the fluorescent NO indicator diaminofluorescein (DAF)-2DA. But

opposite findings have also been reported. In *Vicia faba*, NO promoted stomatal opening, not closure (Sakihama *et al.*, 2003). Since DAF fluorescence was diminished by NOS inhibitors, it was originally suggested that NO in guard cells would be produced by NOS (Garcia-Mata & Lamattina, 2001; Neill *et al.*, 2002; Kolla & Raghavendra, 2007), and this was confirmed by work with the NOS1-deficient *Arabidopsis* mutant, Atnos1 (Guo *et al.*, 2003). However, as mentioned above, NOS1 was later shown not to be an NOS (Crawford *et al.*, 2006; Zemojtel *et al.*, 2006). It is as yet unclear why the NOS1-deficient mutants had a differential stomatal response than WT, and the existence and role of an NOS-like activity for NO production in plants in general, and specifically for stomatal movement are not at all sure at this point. NR has also been considered a source for NO in stomata, which might be the more important as evidence for NOS is again lacking. Using *Arabidopsis nia1/nia2* double mutants with NR activity neither in the mesophyll nor in the stomata, ABA-induced stomatal closure was absent (Desikan *et al.*, 2002), which would confirm that NR is important for stomatal function.

6.5.3 Seed dormancy and germination

N availability is among the most important single factors dominating plant growth, and early information to the seeds about N availability may seem advantageous. Seed dormancy is well-known to be broken by nitrate (Bethke *et al.*, 2006b; Giba *et al.*, 2007). Nitrate or nitrite themselves may be directly sensed, but external NO from nitrifying and denitrifying soil microorganisms, or endogenous NO produced during early stages of germination, appears to be also involved. Passing NO gas over dormant *Arabidopsis* seeds increased the percentage of germinating seeds more than expected from a pure effect of nitrite or nitrate produced by NO oxidation (Libourel *et al.*, 2006). NO may also be produced inside the seed cells or by acidic conversion of nitrite to NO in the apoplast (Bethke *et al.*, 2004). NO contents in embryonic axes of germinating seeds as determined by EPR were elevated sometime after imbibition. Both, an NOS-like activity and NR appeared to contribute to this elevated NO production (Giba *et al.*, 2007, and literature cited; Simontacchi *et al.*, 2007). As the level of NR expression itself is (indirectly) controlled by soil nitrate, NR should actually be better suited than NOS to transform soil nitrate availability into an NO signal. As mentioned above, the existence of NOS in plants is now (again) questionable, and accordingly NR may be a decisive endogenous NO source also during seed germination.

6.5.4 Pathogen defence

Numerous publications have suggested that NO is an important intermediary signal triggering plant defence reactions against pathogens, specifically within the reaction chain leading to the hypersensitive response (HR) (Noritake *et al.*, 1996; Delledonne *et al.*, 1998; Durner *et al.*, 1998; Delledonne

et al., 2001; Yamamoto *et al.*, 2003; Wendehenne *et al.*, 2004; De Stefano *et al.*, 2007). In fact, NO appears to mediate expression of many genes involved in signal transduction and disease resistance (Polverari *et al.*, 2003). NOS-like activity has been mostly considered as the source of NO, based on effects of NOS inhibitors (Foissner *et al.*, 2000; Lamotte *et al.*, 2004). This view can be no longer maintained since both the P-protein and AtNOS1 are no longer considered as plant NO synthases (see above). Thus, the potential participation of NR in NO defence signalling has gained new attention. Indeed, the HR of NR-deficient (*nia1, nia2*) *Arabidopsis thaliana*, challenged with an avirulent strain of *Pseudomonas syringae*, was strongly impaired compared to WT, suggesting a requirement for NR (Modolo *et al.*, 2005). NR was shown to be induced by pathogen signals (Yamamoto *et al.*, 2003), and in *Nicotiana benthamiana*, INF1, a major elicitin secreted by *Phytophtora infestans*, elevated NO production in protoplasts from WT leaves, but not in protoplasts from NR-silenced leaves (Yamamoto-Katou *et al.*, 2006). On the other hand, *nia* double mutants of tobacco, treated with the peptide elicitor cryptogein from the oomycete *Phytophthora cryptogea*, still developed lesions and had increased PR1 expression, both indicative for an HR (Planchet *et al.*, 2006). However, the response of the mutants was somewhat slower and weaker than that of WT (Planchet *et al.*, 2006), which may or may not be related to lack of NO production. Unfortunately, *nia* mutants grown on ammonium N differ in many other respects from nitrate-grown plants. Preliminary experiments to rescue the weaker HR in *nia* double mutants by flushing the leaves with NO were unsuccessful so far (W.M. Kaiser, unpublished). Further, illuminated leaves of the above-mentioned NiR-deficient clone 271, which produce NO in large excess over WT, also gave a normal HR (Planchet *et al.*, 2006), indicating that even a 100-fold NO excess (over WT) does not really affect the development of the HR.

6.5.5 Senescence

In his booklet on 'nitric oxide in plants', Leshem (2000) pointed out that NO could act as an antagonist of ethylene in fruit ripening and senescence. For example, the post-harvest life of strawberry was considerably extended by fumigation with NO. In that context, it is a new and important observation that strobilurin-derived fungicides, aside of their fungicidal action, impair mitochondrial respiration and thereby activate NR in the dark (Glaab & Kaiser, 1999). At the same time, ethylene biosynthesis was impaired, NO emission was increased, senescence was delayed and yields were increased (Köhle *et al.*, 2002). Although the causal connection between all these events is by no means established, they suggest a role of NR-derived NO in the control of senescence and fruit ripening. They also open the possibility for a commercial application by controlling these processes via chemicals, modifying respiration and NR activity.

6.6 Conclusions and open questions

Although NR is the best established enzymatic source for NO in plants, its role in NO signalling is still not clear. *Nia* mutants actually offer excellent tools to test for a role of NR-derived NO. But usually they differ from WT, not only in the capacity to produce NO, but also in many other respects. For example, tobacco mutants and WT often show differences in sugar and amino acid concentrations or in inorganic cation and anion concentrations (H. Yamasaki, unpublished results). This may not only affect growth and vitality of invading pathogens, but may also modulate the response of the plant cells to elicitors or PAMPs. Rescue experiments may offer a solution. If an impaired or modified response of *nia* mutants can be rapidly reversed by flushing the plants with NO gas in realistic concentrations and for moderate times (few hours only), this will be a strong argument for NR-derived NO. Exposing cells or tissues directly to NO donors, which has been frequently used, appears less suitable because all these compounds may have side effects and NO production is not exactly controlled. One of the unsolved basic problems still is that we do not know 'realistic' NO concentrations within tissues and cells, and neither the reaction velocity of NO with cellular constituents nor its autoxidation rates in living cells have been quantified and cannot be estimated as long as the cellular NO concentrations are not known. Another problem lies in the fact that the existence of a plant NOS is still questionable. Plants can exist without any nitrate reduction, in specific natural environments, using ammonium or organic N sources. Accordingly, NR is not obligate as an NO source even in above-ground organs that could not receive nitrite or NO from soil micro-organisms. Thus, without endogenous sources for oxidative NO formation (from L-arginine or from hydroxylamines), the many proposed roles of NO in plants in general would be doubtful.

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Chapter 7

NITRIC OXIDE SIGNALLING IN PLANTS: CROSS-TALK WITH Ca^{2+}, PROTEIN KINASES AND REACTIVE OXYGEN SPECIES

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Abstract: Nitric oxide (NO) is a gaseous free radical recognized as a ubiquitous signal transducer that contributes to various biological processes in animals. It exerts most of its effects by regulating the activities of various proteins including Ca^{2+} channels, protein kinases and transcription factors. In plants, studies conducted over the past 10 years revealed that NO also functions as an endogenous mediator in diverse physiological processes ranging from root development to stomatal closure. Its biological role as an intracellular plant messenger molecule, however, remains poorly understood. Here, we review the molecular basis of NO signalling in animals and discuss current knowledge of NO signalling in plants, focusing on its interplay with Ca^{2+}, protein kinases and reactive oxygen species which are well established as widespread key regulators of signal transduction.

Keywords: calcium; cell death; nitric oxide; protein kinases; reactive oxygen species; signalling

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Nitric oxide (NO) is a noxious free radical gas, which, in the late 1980s, was discovered to exist physiologically in mammalian systems. This discovery offered fresh perspectives on main processes including neurotransmission, immunity and relaxation of vascular smooth muscles (Schmidt & Walter, 1994). Notably, the idea that a simple gas could act as a messenger revolutionized researchers' understanding of signal transduction. Recently, NO was also shown to mediate diverse plant physiological processes such as germination, root growth, flowering, stomatal closure and resistance to biotic as well as abiotic stresses (see reviews by Lamattina *et al*., 2003; Delledonne, 2005; Besson-Bard *et al*., 2008a; Wilson *et al*., 2008). Although evidences supporting NO as a plant physiological mediator are still growing, its functions at the molecular level remain poorly understood and, in some examples, are subject to controversies. Research conducted over the past years has revealed that NO mediates part of its action in a concerted way with the second messenger Ca^{2+}, protein kinases and reactive oxygen species (ROS). The interplays between these molecules operate in cells challenged by biotic and abiotic stresses and modulate various cellular responses including gene expression and cell death. This review introduces the basic concepts of NO signalling in animals and discusses the mechanisms through which NO exerts its signalling activities in plants with a particular emphasis on Ca^{2+}, protein kinases and ROS signalling.

7.1 Basic concepts of NO signalling in animals

The field of research dedicated to NO signalling in animals has been extraordinary fruitful in the past two decades and has led scientists to introduce new concepts of signal transduction. NO is derived from the amino acid L-arginine by the enzymatic activity of nitric oxide synthase (NOS). Once produced, NO acts predominantly via the post-translational modifications of proteins. Three main processes have been described: *S*-nitrosylation, metal nitrosylation and tyrosine nitration. Well over a hundred proteins susceptible to these NO-dependent post-translational modifications and involved in all major cellular activities have been identified. In this section, we describe the principles of *S*-nitrosylation, metal nitrosylation and tyrosine nitration and discuss how these post-translational protein modifications influence Ca^{2+} and protein kinase signalling. Understanding these signalling concepts should facilitate a comprehensive analysis of the way NO acts as a signal in plants.

7.1.1 Metal nitrosylation

As a radical, NO is capable of donating electrons and therefore reacts with transition metals. Covalent interaction of NO with the centres of iron–sulphur clusters, and with haeme and zinc-finger proteins, leads to an increase or a decline in protein activity. Amongst the proteins regulated through metal

nitrosylation, a well-studied target for NO is soluble guanylate cyclase (sGC; Denninger & Marletta, 1999). Soluble guanylate cyclase catalyses the conversion of GTP to pyrophosphate and 3′,5′-cyclic GMP (cGMP), a well-defined second messenger. The interaction of NO with the sGC haeme leads to the opening of the bond between ferrous iron and histidine 105 of the enzyme, thus triggering a conformational change that increases the catalysis of cGMP synthesis by several hundred-fold (Cary *et al.*, 2006; Roy & Garthwaite, 2006). Once produced, cGMP binds to target proteins: cGMP-dependent protein kinases (PKGs), cyclic nucleotide-gated channels (CNGCs) and cyclic nucleotide phosphodiesterases, resulting in cell-specific downstream outputs (Beck *et al.*, 1999). Examples of physiological responses regulated through NO/cGMP signalling include neurotransmission, development, smooth muscle relaxation and blood pressure regulation (Denninger & Marletta, 1999; Krumenacker *et al.*, 2004).

7.1.2 *S*-nitrosylation

S-nitrosylation corresponds to the covalent modification of cysteine sulphurs of proteins by NO (or its derivatives) to form *S*-nitrosothiols (Stamler *et al.*, 2001; Hess *et al.*, 2005). It is not yet clear how NO *S*-nitrosylates target proteins. Candidate mechanisms include the electrophilic attack of the nitrosonium cation (NO^+, resulting from NO auto-oxidation) on thiolate, direct interaction of NO with thiolate in the presence of electron acceptors such as nicotinamide adenine dinucleotide (NAD^+) and complex chemical processes involving nitroxyl anions (NO^-, resulting from NO autoreduction or dinitrogen trioxide decomposition) (Gow *et al.*, 1997; Hanafy *et al.*, 2001; Foster & Stamler, 2004). Interestingly, primary peptide sequences for motifs that might facilitate *S*-nitrosylation have been described, consisting of acidic/basic motifs, as well as hydrophobic motifs surrounding the cysteine residue (Hess *et al.*, 2005; Greco *et al.*, 2006). Similarly to metal nitrosylation, *S*-nitrosylation is a reversible form of post-translational modification. De-*S*-nitrosylation occurs chemically without the help of enzymes or enzymatically through thioredoxin and thioredoxin reductase (Jaffrey *et al.*, 2001; Benhar *et al.*, 2008).

7.1.3 Tyrosine nitration

Tyrosine nitration is mediated by two main NO-derived species including peroxynitrite ($ONOO^-$), resulting from the fast reaction between NO and ROS such as superoxide ($O_2{}^-$), and nitroso-peroxocarboxylate ($ONOOCO_2{}^-$). This adduct is formed following the reaction between $ONOO^-$ and CO_2 at a physiological concentration (Radi, 2004). Nitration occurs in one of the two equivalent carbon atoms in the ortho-position (with respect to the hydroxyl group) of the phenolic ring of tyrosine residues and leads to protein 3-nitrotyrosine (3-NO_2-Tyr) (Radi, 2004). 3-NO_2-Tyr was first related to NO-dependent oxidative stress occurring during inflammatory diseases such as asthma (Schopfer

et al., 2003). Indeed, tyrosine nitration is usually associated with loss of protein functions and target proteins include Mn superoxide dismutase, cytochrome P450, tyrosine hydroxylase, glutamine synthase, glutathione reductase, actin and other cytoskeleton-related proteins (Greenacre & Ischiropoulos, 2001; Gow *et al.*, 2004). It is generally assumed that this process may be irreversible and increase the susceptibility of proteins to degradation by the 20S proteasome (Mannick & Schonhoff, 2002). However, the description of denitrase activities reversing protein nitration in several mammalian tissues suggests that tyrosine nitration might also be a reversible process (Gorg *et al.*, 2007). This latter finding opens the possibility that the formation of 3-NO_2-Tyr may play a role in signal transduction. Regarding this aspect, the relationship with protein tyrosine phosphorylation is particularly noteworthy. Indeed, according to several studies, the importance of tyrosine nitration on cell signalling would lie essentially in the inhibition of tyrosine residues to undergo phosphorylation and/or in the inhibition of phosphatases that allows protein kinases to become dominantly activated (Minetti *et al.*, 2002). A first mechanism has been proposed to explain the activation of tyrosine kinases c-Src by $ONOO^-$: *in vitro* nitration of a C-terminal tyrosine residue could prevent its phosphorylation which normally helps c-Src folding into an inactive conformation (Klotz *et al.*, 2002). At present, however, it is unclear if this process can occur *in vivo*.

7.1.4 Interplays between NO and Ca^{2+}

Currently, NO is recognized as one of the key messengers governing the overall control of Ca^{2+} homeostasis, and almost all types of Ca^{2+} channels and transporters are under its control. The effects of NO on Ca^{2+} channel and transporter activities can be divided into two mechanisms of action: a cGMP-dependent one and a cGMP-independent one. The molecular mechanisms underlying the cGMP-dependent pathway are complex, and at least three processes have been reported. First, cGMP could directly activate CNGCs by virtue of their cyclic nucleotide-binding sites, leading to an enrichment of cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$) (Hanafy *et al.*, 2001; Ahern *et al.*, 2002). Second, the effects of cGMP could be mediated via the activation of PKGs (Clementi & Meldolesi, 1997; Clementi, 1998; Ahern *et al.*, 2002). PKGs have distinct effects on intracellular Ca^{2+}, increasing or decreasing $[Ca^{2+}]_{cyt}$, depending on the target channel, the stimuli and cell types. For example, in hepatocytes, phosphorylation of the inositol 1,4,5-triphosphate (IP_3) receptor by PKGs potentiates IP_3-dependent Ca^{2+} release, whereas an opposite effect is observed in smooth muscle (Clementi, 1998; Murthy & Makhlouf, 1998). Besides the IP_3 receptor, Ca^{2+}-permeable channels and Ca^{2+} transporters whose activities appear to be modulated by PKGs include voltage-dependent Ca^{2+} channels (L-, N-, P/Q- and T-types), store-operated Ca^{2+} channels, mechanosensitive Ca^{2+}-permeable non-selective cation channels,

the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and the plasma membrane Ca^{2+} pump (PMCA) (Clementi, 1998; Wang *et al.*, 2000; Chen *et al.*, 2002; Yao & Huang, 2003; Grassi *et al.*, 2004). The biochemical steps downstream of PKGs that are responsible for the modulation of these channels and transporters have not been completely clarified. Third, to add further complexity to these scenarios, PKG activation has been found to be a crucial step in NO-induced cyclic ADP-ribose (cADPR) synthesis (Willmott *et al.*, 1996; Reyes-Harde *et al.*, 1999; Leckie *et al.*, 2003). cADPR is synthesized from its precursor NAD^+ by ADP-ribosyl cyclase, which might be activated through PKG-induced phosphorylation. cADPR is a Ca^{2+} mobilizing second messenger, which promotes Ca^{2+} release from endoplasmic reticulum in a wide variety of animal cells via the activation of the ryanodine receptors (RYRs) (Fliegert *et al.*, 2007). The cGMP/PKG/cADPR cascade is now recognized as a fundamental mechanism through which NO contributes to the generation and propagation of Ca^{2+} signals in various physiological processes including the induction of hippocampal long-term depression and fertilization in echinoderms (Willmott *et al.*, 1996; Reyes-Harde *et al.*, 1999; Leckie *et al.*, 2003).

The cGMP-independent action of NO on Ca^{2+} homeostasis operates through the direct *S*-nitrosylation of Ca^{2+} channels and transporters. Voltage-dependent Ca^{2+} channels, RYRs, *N*-methyl-D-aspartate receptors, transient receptor potential channels (TRPC) and CNGCs were shown to be reversibly *S*-nitrosylated, with activation or inhibition as a consequence (Broillet, 2000; Stamler *et al.*, 2001; Yoshida *et al.*, 2006; Tjong *et al.*, 2007). For instance, the skeletal muscle RyR1 (one of the three isoforms of RYRs) consists of four homologous 565 kDa subunits containing 100 cysteine residues. In the native protein, 50 of these residues appear to be in a reduced state (Aracena-Parks *et al.*, 2006). Remarkably, submicromolar NO concentrations were shown to activate RYR1 by *S*-nitrosylation of a single cysteine (Cys 3635), this reaction occurring only at low (e.g. physiological) pO_2 but not ambient pO_2 (Sun *et al.*, 2003). This specific *S*-nitrosylation reverses RYR1 inhibition by Ca^{2+}/calmodulin (CaM) and may contribute to enhanced RYR1 activity. Interestingly, Cys 3635 can also be *S*-glutathionylated, suggesting that competition between *S*-nitrosylation and *S*-glutathionylation on Cys 3635 may occur in physiological processes (Aracena-Parks *et al.*, 2006). Another remarkable example of the influence of *S*-nitrosylation on Ca^{2+} channel activities concerns TRPC5, one of the seven TRPC homologues in humans. This plasma membrane Ca^{2+} channel was shown to induce Ca^{2+} entry into human embryonic kidney cells in response to NO released by several NO donors (Yoshida *et al.*, 2006). The molecular mechanism underlying NO-dependent TRPC5 activation may involve the nucleophilic attack of nitrosylated Cys 553 by the free sulphydryl group of Cys 558, thus leading to the formation of a disulphide bond between both cysteine residues. The disulphide bond might stabilize the open state of the channel.

The existence of both cGMP-mediated and direct *S*-nitrosylation pathways expands and enriches the possibilities for NO to modulate Ca^{2+}-dependent signalling processes including gene expression (Peunova & Enikolopov, 1993). Furthermore, because NO production by NOS requires an increase in $[Ca^{2+}]_{cyt}$, the ability for NO to attenuate Ca^{2+} influx by inhibiting some types of Ca^{2+} channels and/or to initiate cytosolic free Ca^{2+} removal by activation of SERCA and/or PMCA helps to protect cells from the deleterious effect of NO. The pathophysiological relevance of these processes is outlined in several examples such as the modulation of neuronal excitability or hypertension but has probably paved the way for new roles in normal and disturbed cell functions. From a mechanical point of view, the plasticity of the NO/Ca^{2+} pathways is particularly intriguing when both pathways act on the same channels. Several studies have provided support that the cGMP-dependent pathway generally occurs at low levels of NO whereas *S*-nitrosylation requires higher levels of NO and tends to proceed with slower kinetics than cGMP-induced actions (Denninger & Marletta, 1999; Hanafy *et al.*, 2001). However, this subject is still controversial and the issue is far from settled (Stamler *et al.*, 2001; Hess *et al.*, 2005).

7.2 NO signalling in plants

During the last decade, NO has been recognized as a versatile player in diverse plant physiological processes. Several routes for NO synthesis have been described: non-enzymatic as well as enzymatic pathways involving nitrate reductase and putative NOS-like enzymes (Kaiser & Huber, 2001; Corpas *et al.*, 2006; Crawford, 2006; Besson-Bard *et al.*, 2008a, 2008b, 2008c; Wilson *et al.*, 2008). A major and still open question concerns the molecular mechanisms of its signalling action. More than hundred proteins have been asserted to undergo regulation by *S*-nitrosylation and metal nitrosylation. Similarly, numerous genes up- and/or down-regulated in response to artificially produced NO have been identified based on microarray analyses. However, with few exceptions, the physiological significance of these post-transcriptional and post-translational modifications remains to be established. Parallel to these approaches, over the last years, evidence gathered from a number of studies has indicated that NO mediates part of its effects through the mobilization of free Ca^{2+}, via the modulation of protein kinase activities and by interacting with ROS. The aim of this section is to concentrate on the interplay between NO, Ca^{2+}, protein kinases and ROS and to describe what is known thus far concerning the physiological impact of the cross-talk between these signalling components.

7.2.1 Interplays between NO and Ca^{2+}

A large number of signals, including plant hormones, light, biotic as well as abiotic stresses, cause transient and specific changes in intracellular $[Ca^{2+}]$.

In the recent years, it has become increasingly appreciated that the signalling components that govern these changes include NO. Furthermore, because NO production is under the control of intracellular Ca^{2+} fluctuations, NO might also act as a Ca^{2+} sensor contributing to decoding the intracellular Ca^{2+} changes in plants.

7.2.2 NO acts as a Ca^{2+}-mobilizing messenger

The first conclusive evidence implicating NO as a Ca^{2+}-mobilizing messenger in plant cells came from studies exploring the ability of NO donors to induce increases in intracellular [Ca^{2+}]. Using Ca^{2+}-sensitive dye fura-2 fluorescence ratio imaging, Garcia-Mata *et al.* (2003) showed that treating *Vicia faba* guard cells by the NO donor *S*-nitroso-*N*-acetyl-penicillamine (SNAP) induced stomatal closure by promoting an increase in intracellular [Ca^{2+}]. The ability of exogenous NO to induce a rise of intracellular [Ca^{2+}] was further supported by the finding that the NO donor diethylamine NONOate (DEA/NO) triggers a transient rise of $[Ca^{2+}]_{cyt}$ in transgenic *Nicotiana plumbaginifolia* cell suspensions, expressing the Ca^{2+}-reporter apo-aequorin (Lamotte *et al.*, 2004, 2006). By contrast, the same NO donor did not induce any change in nuclear free Ca^{2+} concentration ($[Ca^{2+}]_{nuc}$) (Lecourieux *et al.*, 2005), suggesting that NO effects on Ca^{2+} homeostasis are restricted to specific cellular compartments.

The influence of NO on the cellular [Ca^{2+}] in physiological contexts was highlighted by the demonstration that NO scavengers and mammalian NOS inhibitors reduced stimulus-induced rises in $[Ca^{2+}]_{cyt}$. Notably, Lamotte *et al.* (2004) showed that the NO scavenger cPTIO and NOS inhibitors reduced the $[Ca^{2+}]_{cyt}$ increases induced in *N. plumbaginifolia* cells by the proteinaceous elicitor cryptogein, secreted by the oomycete *Phytophthora cryptogea*. These pharmacological agents did not reduce the cryptogein-triggered $[Ca^{2+}]_{nuc}$ rises, confirming the observation made using NO donors (see above; Lecourieux *et al.*, 2005). Similar effects of NO were reported in grapevine cell suspensions exposed to the elicitor endopolygalacturonase 1 from *Botrytis cinerea* (Vandelle *et al.*, 2006). Another example emerges from studies investigating NO function in plant cells exposed to hyperosmotic stress. Under this condition, addition of the NO scavenger cPTIO reduced the increase in $[Ca^{2+}]_{cyt}$ in *N. plumbaginifolia* cell suspensions, expressing the calcium reporter apo-aequorin, highlighting again the role of NO in $[Ca^{2+}]_{cyt}$ elevations (Gould *et al.*, 2003; Lamotte *et al.*, 2006).

7.2.3 Underlying mechanisms

The mechanisms that enable NO-triggered changes in intracellular [Ca^{2+}] have been intensively studied in recent years. Pharmacological analyses of [Ca^{2+}] variations have indicated that NO might activate both plasma membrane and intracellular Ca^{2+}-permeable channels (Garcia-Mata *et al.*, 2003; Lamotte *et al.*, 2004, 2006; Lecourieux *et al.*, 2005; Vandelle *et al.*, 2006). Whereas

the putative identity of the NO-sensitive plasma membrane Ca^{2+}-permeable channels remains unknown, several lines of evidence have suggested that NO might target RYR-like channels. Indeed, the increase in $[Ca^{2+}]_{cyt}$ of *N. plumbaginifolia* cells treated by the NO donor DEA/NO was sensitive to ruthenium red, an inhibitor of mammalian RYRs (Lamotte *et al.*, 2004). A similar inhibitory effect was obtained using the cADPR antagonist 8Br-cADPR, designing cADPR as possible intermediate of the NO signal leading to changes in intracellular $[Ca^{2+}]$. According to Garcia Mata *et al.* (2003), cADPR might function together with cGMP as reported in animals (Willmott *et al.*, 1996; Reyes-Harde *et al.*, 1999). Indeed, they showed that ryanodine, an antagonist of RYR as well as ODQ (1-*H*-(1,2,4)-oxadiazole-(4,3-*a*)quinolxalin-1-one), an inhibitor of sGC, was able to suppress SNAP-mediated increase in intracellular $[Ca^{2+}]$ in *V. faba* guard cells. The authors provided several arguments indicating that a similar NO/cGMP/cADPR/Ca^{2+} pathway might occur in response to abscisic acid (ABA).

Besides cADPR and cGMP, evidences that NO could also contribute to $[Ca^{2+}]_{cyt}$ increases via phosphorylation events were provided. Indeed, protein kinase inhibitors efficiently suppress NO donor-triggered elevation in $[Ca^{2+}]_{cyt}$ in *V. faba* guard cells and *N. plumbaginifolia* cells (Sokolovski *et al.*, 2005; Lamotte *et al.*, 2006), indicating that the signalling cascades relaying NO and Ca^{2+}-permeable channels could involve protein kinases, besides or together with cADPR. At present, only one protein kinase candidate, named NtOSAK (*Nicotiana tabacum* osmotic stress-activated protein kinase), has been identified (Lamotte *et al.*, 2006). This aspect is discussed farther. Finally, it should be specified that several arguments pointing out the involvement of NO in stimulus-induced plasma membrane depolarization have been reported (Lamotte *et al.*, 2006; Vandelle *et al.*, 2006). Such NO-dependent changes of the plasma membrane potential might modulate the activity of plasma membrane Ca^{2+}-permeable channels. The mechanisms underlying this effect are poorly understood.

7.2.4 Impacts of the NO/Ca^{2+} pathways

The first conclusive evidence of the biological significance of an NO/Ca^{2+} pathway came from studies in which the function of NO in plant defence responses was investigated (Durner *et al.*, 1998; Klessig *et al.*, 2000). Using *N. tabacum* plants and suspension cells treated with a recombinant mammalian NOS or NO donors, respectively, it was shown that NO was able to mediate the expression of the defence-related genes *PR* (*pathogenesis related*)-1 and *PAL* (*phenylalanine ammonia lyase*) through cGMP and/or cADPR. In addition to gene expression, compelling evidence suggests a role for the interplay between NO and Ca^{2+} on micro-organism-triggered hypersensitive response (HR). In this context, NO appears to act as a Ca^{2+} sensor, contributing to decode the intracellular Ca^{2+} changes in plants leading to cell death. Both pharmacological and genetic experimental data support this concept (Delledonne

et al., 1998; Lamotte *et al.*, 2004; Ali *et al.*, 2007). For instance, in cryptogein-elicited tobacco cell suspensions, NO production is stimulated by an influx of extracellular Ca^{2+} (Lamotte *et al.*, 2004). In turn, NO partly contributes to the elicitor-triggered cell death. The plasma membrane cyclic nucleotide-gated Ca^{2+}-permeable channel CNGC2 was identified as one of the putative key components of this pathway in *Arabidopsis thaliana* (Ali *et al.*, 2007). Accordingly, the HR normally suppressed in the *A. thaliana dnd1* (*defence no death 1*) mutant, impaired in CNGC2 expression, was shown to be partially restored by the NO donor sodium nitroprusside (SNP). Further research is needed to understand how NO, the production of which is stimulated by an influx of Ca^{2+}, can contribute to HR. Because, as discussed previously, NO also amplifies the mobilization of free Ca^{2+}, it is possible that the NO-dependent rise of intracellular $[Ca^{2+}]$ facilitates cellular Ca^{2+} overload, which, in turn, could cause cytotoxicity and could trigger cell death. Besides mediating defence responses, the NO/Ca^{2+} pathways might influence diverse cellular processes such as ABA-induced stomatal closing or auxin-mediated adventitious root formation (Garcia-Mata *et al.*, 2003; Lamattina *et al.*, 2003; Desikan *et al.*, 2004).

7.2.5 Interplays between NO and protein kinases

Multiple protein kinase cascades are involved in the transduction and amplification of the information received through cellular signals. Protein phosphorylation and dephosphorylation are very common intracellular signalling modes. Kinases and phosphatases regulate a wide range of cellular processes such as enzyme activation, assembly of macromolecules, protein localization and degradation. In animals, NO has been described to modify the activity of protein kinases involved in signal transduction, such as mitogen-activated protein kinase (MAPK) cascades, Janus kinases or protein kinase C (Beck *et al.*, 1999). Also, the activity of primary metabolism-related kinases, for instance pyruvate kinase, was identified to be modified by *S*-nitrosylation (Gao *et al.*, 2005).

It is presently known that serine/threonine protein kinases play a crucial role in the transduction of various extra- and intracellular signals in plants (Mishra *et al.*, 2006). However, although the identification of NO-modulated protein kinases is a major issue in the understanding of NO-dependent signal transduction, only few of them have been identified and studied. Moreover, these observations have been carried out using, for most part of them, artificially generated NO from NO donors, and not during a physiological plant process.

7.2.6 NO modulates MAPK activities

In all eukaryotes, MAPK pathways serve as highly conserved central regulators of growth, death, differentiation, proliferation and stress responses (Samaj *et al.*, 2004; Qi & Elion, 2005). MAPKs form the terminal components

of the MAPK cascades (MAPKKK → MAPKK → MAPK). MAPKs are activated by MAPK kinases (MAPKKs/MEKs) via dual phosphorylation of conserved threonine and tyrosine residues in the motif TxY located in the activation loop. Some evidence shows that in plants NO also contributes to the activation of MAPK cascades.

Kumar and Klessig (2000) found an MAPK activated by NO in tobacco leaves and cell suspensions. Injection of tobacco leaves with recombinant rat neuronal NOS, together with its co-factors and substrate, transiently activated a 48 kDa protein kinase phosphorylating MBP (myelin basic protein), an artificial MAPK substrate. Using the specific anti-SIPK antibody in immunocomplex kinase activity assay, they identified this kinase as salicylic acid (SA)-induced protein kinase (SIPK). The NO donors – *S*-nitroso-L-glutathione (GSNO), DEA/NO and SNAP – also transiently activate SIPK in tobacco cell suspension cultures (Kumar & Klessig, 2000; Besson-Bard *et al.*, 2008b). Depending on the NO-generating system, this activation was shown to be SA-dependent or SA-independent.

Although SIPK is the first and the only NO-dependent MAPK identified to date, there are more data indicating influence of NO on MAPK pathways. Working with *A. thaliana* shoots, Capone *et al.* (2004) demonstrated that brief oxidative or nitrosative stresses in the roots, using, respectively, H_2O_2 and the NO donor SNP, triggered the activation of a 38 kDa protein kinase able to phosphorylate MBP. It was confirmed that this kinase belongs to the MAPK family by using antibodies raised against the active (phosphorylated) form of a mammalian p38 MAPK, but no direct relation between this activation and NO production has been clearly demonstrated. Another example of NO ability to activate MAPK was provided by Clarke *et al.* (2000). The authors reported that a 47 kDa protein kinase, able to phosphorylate MBP, is activated within 5 minutes in response to the NO donor Roussin's black salt in *A. thaliana* cell suspensions. A role for the 47 kDa protein kinase in mediating NO-induced cell death was tentatively assigned. However, pharmacological inhibition of this MAPK was inefficient in reducing cell death. Thus, many questions regarding the cellular impact of the activation of this MAPK by NO remain opened.

Recently, a functional link between NO and MAPKs has been established in ABA signalling in mesophyll cells of maize leaves (Zhang *et al.*, 2007). Using pharmacological approach, a linear interplay of these signalling components has been demonstrated: ABA treatment induces H_2O_2 production, acting upstream NO synthesis. In turn, NO favours the activation of a 46 kDa MAPK. Induction of this MAPK results in an enhancement of the expression of gene-encoding antioxidant proteins such as catalase, superoxide dismutase, glutathione reductase or ascorbate peroxidase, thus improving the total antioxidative activity of the cells. This cascade of reactions could be triggered in response to stresses such as water stress, thus highlighting a key role for NO in controlling MAPK involved in the plant adaptive response to abiotic stresses.

Finally, several lines of evidence suggest that NO and MAPKs act together in the auxin transduction pathway leading to adventitious root formation. More precisely, pharmacological-based experiments designed NO as a key regulator of an auxin-induced 48 kDa MAPK, sensitive to the MAPK inhibitor PD098059 (Pagnussat *et al.*, 2004). Cucumber explants co-treated with an NO donor and PD098059 showed a significant reduction in root length and root number, demonstrating firstly that NO is required for the activation of the 48 kDa MAPK, and secondly that this activation is essential for adventitious root formation. Interestingly, addition of an sGC inhibitor was not able to prevent the NO-dependent activation of the 48 kDa MAPK activation. This latter result suggested that this MAPK might be part of an NO-dependent/cGMP-independent signalling pathway, which parallels a previously characterized NO/cGMP-dependent signalling cascade also acting in auxin-induced adventitious root formation (Pagnussat *et al.*, 2003).

7.2.7 NO and Ca^{2+}-dependent protein kinases

Support for the hypothesis that NO promotes the activation of Ca^{2+}-dependent protein kinases (CDPKs) came through biochemical and pharmacological approaches that showed the ability of SNP and auxin to induce the activation of a 50 kDa protein kinase in a Ca^{2+}-dependent manner in cucumber hypocotyls (Lanteri *et al.*, 2006). The auxin- or SNP-triggered activation of the 50 kDa protein kinase was also reduced by CaM antagonists including trifluoperazine dihydrochloride (TFP) and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide hydrochloride (W-7). These results let to the assumption that the 50 kDa protein kinase may contain CaM-like Ca^{2+}-binding domains, a structural feature of CDPKs. However, it should be noticed that both TFP and W-7 are not specific inhibitors of CDPKs. Indeed, these compounds also affect the binding of Ca^{2+} to proteins such as CaM and calcineurin B-like proteins (Anil & Rao, 2000) which normally regulate the activity, and therefore the function, of a variety of target proteins including protein kinases (e.g. CcaMKs, CaMKs and SnRKs3; Hrabak *et al.*, 2003).

At a physiological level, the CaM antagonists TFP and W-7 were shown to negatively affect NO- or auxin-induced adventitious root formation in cucumber, suggesting the involvement of the 50 kDa protein kinase in this process (Lanteri *et al.*, 2006). Interestingly, in contrast to the NO-dependent 48 kDa MAPK described above, the activity of the 50 kDa putative CDPK triggered by SNP or auxin was inhibited by sGC inhibitors, suggesting that this protein kinase is part of the NO/cGMP-dependent pathway leading to adventitious root formation (Pagnussat *et al.*, 2003). Because the activity of the 50 kDa cucumber CDPK was detected at the earlier stages of adventitious root formation, it was proposed that this NO-dependent protein kinase can be involved in cell dedifferentiation, division and/or differentiation (Lanteri *et al.*, 2006).

7.2.8 NO and SnRKs

Plant SNF1 (sucrose non-fermenting 1)-related protein kinases (SnRKs) are classified into three subfamilies: SnRK1, SnRK2 and SnRK3. Available evidence indicates that SnRK1 might play an important role in the regulation of global metabolism, the disturbance of which might lead to developmental or adaptation defects (for reviews, see Halford & Hardie, 1998; Halford *et al.*, 2003; Hrabak *et al.*, 2003). The SnRK2 and SnRK3 subfamilies are specific to plants and are involved in environmental stress signalling (for reviews, see Hrabak *et al.*, 2003; Boudsocq & Lauriere, 2005).

First evidence that NO modulates the activity of SnRKs was provided by Lamotte *et al.* (2006). These authors showed that application of the NO donor DEA/NO to tobacco cell suspensions resulted in a fast and transient activation of a 42 kDa protein kinase phosphorylating MBP and histone IIIS, another protein kinase substrate. Using specific antibodies in immuno-complex activity assay, this 42 kDa protein kinase was identified as NtOSAK, a member of the SnRK2 family (Kelner *et al.*, 2004). Similarly to other SnRK2 members in *A. thaliana* (Droillard *et al.*, 2002; Boudsocq *et al.*, 2004) and rice (Kobayashi *et al.*, 2004), NtOSAK is activated within minutes in response to hyperosmotic stress (Mikolajczyk *et al.*, 2000), a process which leads to a rapid increase in NO synthesis (Gould *et al.*, 2003). Importantly, NtOSAK activation in response to osmotic stress was abolished by the NO scavenger cPTIO, highlighting the ability of NO in promoting SnRK2 activation during physiological processes.

These findings might be of general importance because it is consistent with the central roles of both NO and SnRK2s in the regulation of stomatal closure as well as defence responses. This assumption is exemplified by the involvement of NO and the *A. thaliana* SnRK2 protein kinase OST1 (open stomata 1) in common pathways. Indeed, OST1 was found to mediate the regulation of stomatal closure by ABA, a function also assigned to NO (Mustilli *et al.*, 2002). Similarly, the pathogen-associated molecular pattern flagellin 22 and lipopolysaccharides were shown to trigger stomatal closure through an NO- and OST1-dependent signalling cascade (Melotto *et al.*, 2006). Although a mechanistic connection between NO and OST1 remains to be established, these data further support the hypothesis that NO and SnRK2 protein kinases act together in the plant adaptive responses to biotic as well as abiotic stresses.

7.3 Interplays between NO and ROS

7.3.1 Impacts of the NO/ROS balance in HR

The interplay between ROS and NO has long been recognized in the animal field (Curtin *et al.*, 2002). In fact, many of the NO-derived responses are believed to stem from the reaction between NO and ROS to form reactive

nitrogen species (RNS) such as $ONOO^-$. In contrast to ROS, NO is exclusively produced by specific enzymes in animal cells (Turpaev & Litvinov, 2004). Conversely, plants can produce NO through a number of pathways, both enzymatic or non-enzymatic (Kaiser & Huber, 2001; Corpas *et al.*, 2006; Crawford *et al.*, 2006; Besson-Bard *et al.*, 2008a, 2008b, 2008c; Wilson *et al.*, 2008). An interaction between both molecules during the HR was originally suggested by Delledonne *et al.* (1998, 2001). The authors observed a strong NO burst accompanied by ROS generation following inoculation of soybean cell cultures with avirulent bacteria. However, up-regulation of NO following bacterial infection was insufficient to activate the HR cell death. Moreover, $ONOO^-$ is unable to induce cell death in plant cells. Thus, although O_2^- is not directly involved in this response, its conversion to H_2O_2 by superoxide dismutase is critical for the H_2O_2/NO signalling pathway.

De Pinto *et al.* (2002, 2006) also demonstrated that the NO/ROS couple is necessary for programmed cell death (PCD) in *N. tabacum* cv. BY-2 cells and a strong spatio-temporal correlation was reported between ROS and NO production during powdery mildew-dependent HR in barley (Mur *et al.*, 2008). The HR elicited by *Pseudomonas syringae* pv. *phaseolicola* and pv. *tomato* harbouring the *avrRpm1* gene in tobacco and *A. thaliana* was preceded by an NO peak followed immediately by an H_2O_2 burst (Mur *et al.*, 2005). Similarly, inoculation of *A. thaliana* cell cultures with *P. syringae* pv. *maculicola* carrying the *avrRpm1* avirulence gene resulted in a rapid and sustained NO increase, whereas the increased production of H_2O_2 was delayed (Clarke *et al.*, 2000). Thus, although some discrepancies exist in the literature concerning the cooperation between NO and H_2O_2, the NO/H_2O_2 balance still seems crucial for many HR-dependent cell death events.

The question may thus arise as to how do cellular NO and H_2O_2 interact during the HR. A mechanistic answer was provided by Romero-Puertas *et al.* (2007). Employing a proteomic strategy based on the biotin-switch assay, the authors identified several proteins in which the *S*-nitrosylation level is increased in *A. thaliana* leaves challenged by the incompatible pathogen *P. syringae*. The authors focused their attention on peroxiredoxin II E (PrxII E), a member of the peroxiredoxin family which catalyses the reduction of H_2O_2 but also $ONOO^-$, depending on the isoforms. Extensive biochemical and genetic approaches indicate that *S*-nitrosylation of PrxII E inhibits its capacity to detoxify $ONOO^-$. Based on these data, an interesting model was proposed in which *S*-nitrosylation of PrxII E impairs its peroxynitrite reductase activity, thus leading to an increased level of tyrosine nitration, a hallmark of NO/ROS-dependent oxidative stress.

7.3.2 Candidate sites of interaction between NO and ROS during the HR

In animals, mitochondria play a central role in PCD by releasing cytochrome *c* and activating caspases, and there is growing belief that the intracellular

redox status is critical in mitochondria-dependent cell death in animals (Kowaltowski *et al.*, 2001). In particular, the interaction between mitochondrial cytochrome *c* and NO constitutes an important signalling pathway for the controlled production of H_2O_2 (Brookes *et al.*, 2002). In plants, mitochondria have been identified as key players of cell redox homeostasis and signalling (Noctor *et al.*, 2007), as well as important integrators of PCD (Jones, 2000; Swidzinski *et al.*, 2002, 2004; Lam, 2004). Transgenic tobacco cells lacking the alternative oxidase show enhanced susceptibility to various cell death inducers, including H_2O_2 (Robson & Vanlerberghe, 2002; Vanlerberghe *et al.*, 2002), and H_2O_2-driven cell death occurs through a mitochondria-dependent pathway (Mur *et al.*, 2008). Interestingly, mitochondria are also considered as potential sites of NO action. Notably, although oxygen consumption via the cytochrome pathway is inhibited by NO in isolated soybean cotyledons (Millar & Day, 1996) and carrot cell suspensions (Zottini *et al.*, 2002), in both cases the cyanide-insensitive alternative oxidase is not significantly affected. Similarly, Yamasaki *et al.* (2001) found that the alternative pathway is resistant to NO in plant mitochondria isolated from mung bean. Thus, these data suggest that the NO effect on the respiratory pathway may play some role in maintaining mitochondrial homeostasis by limiting ROS release. Further support for this comes from the fact that mitochondria can support nitrite-dependent NO synthesis (Planchet *et al.*, 2005) and that AtNOA1 (nitric oxide associated 1), an enzyme initially thought to display NOS activity, is targeted to the mitochondria (Guo & Crawford, 2005). Considering the hydrophobic and diffusible nature of NO, these data provide additional support for a potential interaction between NO and ROS in the mitochondria or its vicinity, thus potentially participating in mitochondria-derived cell death signals.

The chloroplast has also been put forward as a critical player in the development of the HR under light (Zeier *et al.*, 2004; Montillet *et al.*, 2005; Mur *et al.*, 2008). NO can inhibit chloroplast electron transport in a reversible manner (Takahashi & Yamasaki, 2002), and chloroplasts have also been identified as a potential participant in NO synthesis and $ONOO^-$ production in plants (Gould *et al.*, 2003; Jasid *et al.*, 2006). In fact, a strong correlation between $ONOO^-$ and the presence of oxidatively modified proteins in both the stroma and the thylakoids was observed in soybean chloroplasts (Jasid *et al.*, 2006). Furthermore, it was suggested that $ONOO^-$ interacts with non-haeme Fe^{2+}, leading to PSII inhibition on the acceptor site (Gonzalez-Perez *et al.*, 2008). Thus, *in situ* production of NO in the chloroplasts could play a protective role in preventing oxidation of chloroplast lipids and proteins, but alternatively, the reaction between $O_2\cdot^-$ and NO could lead to $ONOO^-$ production, which could be responsible for the impairment of the photosynthetic machinery. Thus, the different HR cell death phenotypes observed in the light or in the dark during pathogen infection (Montillet *et al.*, 2005) may therefore depend not only on the effect of NO on chloroplast homeostasis but also on the release of ROS by the chloroplast and their interaction with NO.

Finally, both ROS and NO are also produced by peroxisomes. Peroxisomes are a major site of $O_2\cdot^-$ and H_2O_2 production in plant cells (del Rio *et al.*, 2002). They have also been considered as a major site of NO synthesis (Corpas *et al.*, 2001). In a study, the possible interaction between NO and peroxisomal H_2O_2 production on gene regulation was analysed in transgenic catalase antisense tobacco plants (Zago *et al.*, 2006). The different phenotypes obtained under various concentrations of H_2O_2 and NO clearly supported the idea that a tight balance between both molecules is necessary for HR-type cell death. Furthermore, this cDNA-AFLP analysis demonstrated that only 16 differentially expressed transcripts required both NO and H_2O_2. In contrast, 152 genes could be modulated by either NO or H_2O_2, thus demonstrating that the NO and H_2O_2 pathways may overlap to a greater extent than initially thought in HR-induced cell death.

7.3.3 A protective molecule

Finally, NO has been reported as both a cytotoxic and cytoprotective molecule in plants (Beligni & Lamattina, 2001). This dual role may depend to a large extent on a tight spatio-temporal kinetic of cellular concentrations, which will be governed by the production, displacement and removal of RNS (Noriega *et al.*, 2007). NO was shown to function as an antioxidant and thus to protect plants from a variety of abiotic stresses such as drought, heat, salt or heavy metal stresses (Garcia-Mata & Lamattina, 2002; Uchida *et al.*, 2002; Noriega *et al.*, 2007; Tewari *et al.*, 2008) and oxidative stress (Beligni & Lamattina, 2001; Dubovskaya *et al.*, 2007). First, this protective effect may originate from direct detoxification of ROS by NO. Indeed, it is widely believed that NO can protect cells against oxidative stress by preventing the Fenton reaction by scavenging iron, thus avoiding the formation of hydroxyl radicals, one of the most phytotoxic oxygen radicals (Wink *et al.*, 1995). Furthermore, the reaction between NO and $O_2\cdot^-$, which leads to $ONOO^-$ formation, may help in reducing the adverse effects of oxygen radical accumulation. Second, the antioxidative properties of NO may rely on its ability to alter the plant antioxidant system. Indeed, it was demonstrated that NO could stimulate the expression of a haeme oxygenase, which catalyses the conversion of haeme to biliverdin IX with the concomitant release of CO and iron, and acts against oxidative stress in plants (Noriega *et al.*, 2007). In addition, several studies have also reported an up-regulation of several antioxidant enzymes following treatment with low NO concentrations (Parani *et al.*, 2004; Shi *et al.*, 2005).

Finally, new insight into NO cytoprotective effects was provided by Belenghi *et al.* (2007). These authors reported that the *A. thaliana* metacaspase 9 (AtMC9) is constitutively *S*-nitrosylated *in vivo* at the catalytic Cys 147 residue. This post-translational modification inhibits AtMC9 autoprocessing and proteolytic activity. This mechanism resembles those described for caspase-3 in mammalian cells. In resting cells, *S*-nitrosylation of the catalytic cysteine of caspase-3 maintains the enzyme in an inactive form

(Mitchell *et al.*, 2007). Upon apoptosis inducer action, thioredoxin mediates denitrosylation of mitochondria-associated caspase-3, a process required for caspase-3 activation that promotes apoptosis (Benhar *et al.*, 2008). Whether a similar thioredoxin-dependent de-*S*-nitrosylation contributes to AtMC9 up-regulation in plants remains to be established.

7.4 Conclusion

NO has undoubtedly been an area on intense research over the past years. While the number of physiological processes involving NO is likely to grow, understanding of how this gas exerts its effects at the molecular level is still in its infancy. Clearly, there is no simple and uniform picture of the signalling function of NO (Fig. 7.1). Accumulating evidence now points out NO as one

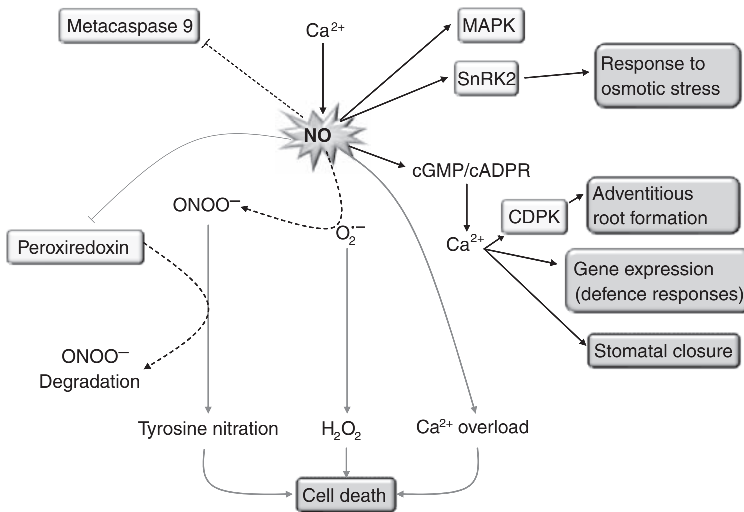

Figure 7.1 Schematic representation of the interplays between NO and Ca^{2+}, protein kinases and ROS in plant cells. NO production is up-regulated by Ca^{2+}-dependent processes. In turn, NO promotes increases in the cytosolic Ca^{2+} concentration through complex pathways involving cADPR and/or cGMP. The resulting rise in the cytosolic Ca^{2+} concentration contributes to CDPK activation and defence gene expression and represents a key signalling step in auxin-induced adventitious root formation and ABA-induced stomatal closure. NO also exerts part of its activities through MAPK and SnRK2. Although several arguments pointed out for a role of the NO/SnRK2 pathway in the plant adaptive response to osmotic stress, the cellular impacts of the NO-dependent activation of MAPK remain enigmatic. NO signalling during cell death (grey arrows) is in part understood by the requirement of H_2O_2 and tyrosine nitration and might involve a Ca^{2+} overload. Tyrosine nitration results as a consequence of peroxiredoxin inhibition through *S*-nitrosylation. Protective principle (dotted arrows) may partly arise from metacaspase 9 *S*-nitrosylation and peroxiredoxin activity.

of the key messengers governing the control of Ca^{2+} homeostasis. The interaction between NO and Ca^{2+} operates in response to various stimuli in plants, suggesting that the cross-talk between both messengers is a basic transduction mechanism as reported in other organisms. Similarly, NO and ROS act in concert with protective or toxic effects as potential consequences, depending on the tight spatio-temporal kinetics of their respective production. It is however extremely difficult to predict the effects of the concerted action of NO and ROS, the main problems facing these studies being the current lack of drugs capable of selectively acting in one species and our limited understanding of NO chemistry in plants. Finally, the ability of NO to modulate protein kinase activities represents another example of how NO mediates its action. The question of the physiological influence of NO/phosphorylation cascades remains, for the most, unanswered.

It is to be hoped that current and future studies will contribute towards the identification of *S*-nitrosylated, metal-nitrosylated and tyrosine-nitrated proteins mediating NO signalling. Deeper insight into these NO-dependent post-translational protein modifications not only will permit the detailed characterization of the biochemical steps involved in NO control of the Ca^{2+}, ROS and protein kinase systems, but also will allow us to understand the physiological significance of the heterogeneous behaviours of NO in plants.

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Chapter 8

THEANINE: ITS OCCURRENCE AND METABOLISM IN TEA

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Abstract: Tea is the second most consumed beverage in the world, after water. It is widely regarded as a healthy beverage, associated with cancer prevention, boosting the immune system and mental alertness, although its popularity as a beverage probably has more to do with its pleasant taste and relaxation benefits. These qualities are attributed to a unique neurologically active non-protein amino acid called L-theanine (γ-ethylamino-L-glutamic acid). In this chapter, the authors review the properties of theanine, its occurrence and metabolism in tea. The possible role of theanine in nitrogen transport is discussed. The regulation of theanine levels is reviewed in the context of plant nitrogen metabolism. Particular emphasis is placed on the identification of enzymes involved in the synthesis and metabolism of theanine. Finally, the authors summarize the status of their endeavours to identify candidate genes contributing to the regulation of theanine levels in tea.

Keywords: ethylamine; γ-glutamyltranspeptidase; glutamate dehydrogenase; glutamic acid; GOGAT; glutaminase; glutamine synthetase; L-theanine

8.1 Introduction

Theanine was first discovered in 1949 by Sakato. It is a water-soluble compound, which rapidly enters the bloodstream following ingestion. In rats, peak plasma concentrations of theanine are found 30 minutes after oral administration (Unno *et al.*, 1999). Yokogoshi *et al.* (1998b) showed (also in rats)

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that theanine crossed the blood–brain barrier via a leucine-preferring transport system.

8.2 Physiological benefits of theanine

One of the effects of theanine in the brain seems to be to stimulate α-brain wave activity. Animals and humans generate weak electric pulses on the surface of their brains, called brain waves. Brain waves are classified into four types – α-, β-, δ- and λ-waves – according to their frequency, and each type is associated with a particular mental condition, as shown in Figure 8.1. α-Brain wave activity is associated with a feeling of relaxation in humans (Juneja *et al.*, 1999), so much so that generation of α-brain waves is sometimes used as an index of relaxation.

In a volunteer study designed to investigate the mental effect of theanine, significantly enhanced α-brain wave activity was observed 40 minutes after intake of theanine relative to water (Juneja *et al.*, 1999).

Despite containing a reasonable amount of caffeine, green tea is often used as a relaxing beverage. In Kakuda *et al.*'s study (2000), it was suggested that theanine had an antagonistic effect on caffeine's stimulatory action, when consumed at similar concentrations. Conversely, the study also indicated a stimulatory effect of theanine when it was given in small doses, such as 1 or 2 μmol/kg body weight, suggesting a dual effect of theanine, depending on its concentration.

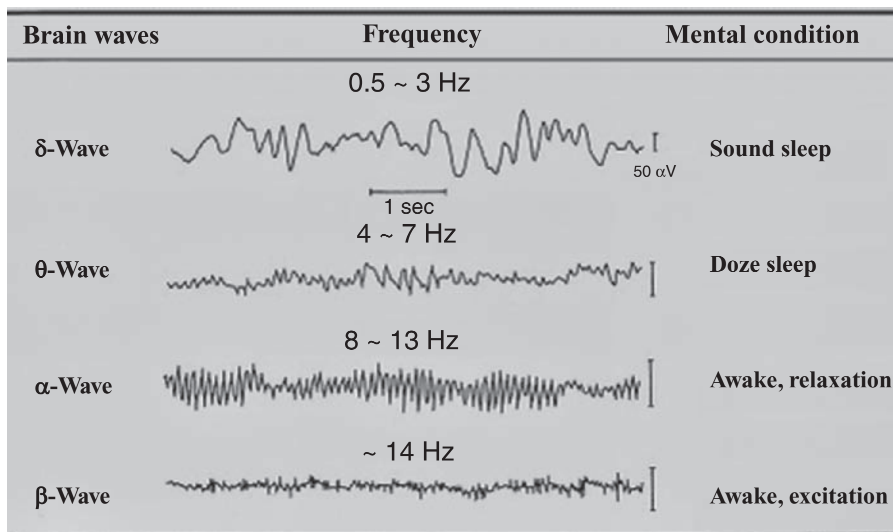


Figure 8.1 Classification of brain waves. Reproduced, with permission, from Juneja *et al.* (1999).

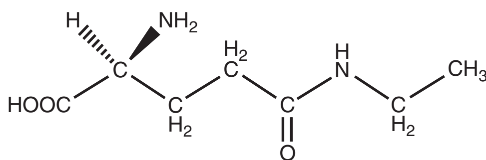
The regulation of blood pressure is known to be partly dependent on the catecholaminergic and serotonergic neurons in both brain and peripheral nervous systems (Sved *et al.*, 1979; Kuhn *et al.*, 1980). As it had been demonstrated that theanine reduced serotonin concentration in the brain, either by decreasing serotonin synthesis or by increasing its degradation (Kimura & Murata, 1986; Yokogoshi *et al.*, 1998a), a study was performed to investigate the effect of theanine on lowering blood pressure using spontaneously hypertensive rats (SHR) (Yokogoshi *et al.*, 1995). In this study, various amounts of theanine were injected into SHR, and the blood pressure was measured before and 60 minutes after administration. L-glutamine, which has a similar structure to theanine, was used as control. The administration of theanine resulted in a significant lowering of blood pressure, whereas glutamine did not show nearly the same antihypertensive effect. The blood pressure-lowering effect of theanine was dose-dependent, with the highest concentration causing the greatest decrease. Theanine may also affect the peripheral nervous system and peripheral blood vessels (Yokogoshi *et al.*, 1995, 1998a), having a calming effect on the mental state by lowering blood pressure (Juneja *et al.*, 1999).

Numerous *in vitro* and animal studies have been performed to investigate the effect of theanine on cancer. For example, theanine decreased the size of ovarian tumours in sarcoma-bearing mice, when it was given jointly with chemotherapeutic agents (Sugiyama & Sadzuka, 1999, 2003, 2004; Sadzuka *et al.*, 2001). Sugiyama and Sadzuka (2004) also proposed a possible route whereby theanine might protect normal cells from damage by chemotherapeutic agents. Once theanine is taken up by normal cells, it is enzymatically converted into glutamate, which increases glutamate concentration and hence GSH levels in normal tissues. The increase in GSH may result in both reduced LPO level and increased DOX efflux from normal cells, and inhibit oxidative damage induced by other drugs.

8.3 Chemical properties and characteristics of theanine in tea

L-theanine is a unique non-protein amino acid found almost exclusively in tea (*Camellia sinensis*). It also appears in trace amounts in two other *Camellia* species (*C. japonica* and *C. sasanqua*) and one species of mushroom (*Xerocomus badius*). Theanine was first discovered by Sakato (1949) in an aqueous extract of green tea leaves. Further work carried out by Cartwright *et al.* (1954) confirmed its existence in tea as well as its high reactivity with ninhydrin. Chemically, theanine was found to be the γ-ethylamine of glutamic acid (Fig. 8.2).

Theanine is the predominant amino acid in tea and accounts for about 50% of the total free amino acids and 1~2% of the dry weight of tea leaves. Along with glutamine and other free amino acids, theanine forms a non-protein nitrogen reserve.



(γ-Ethylamino-L-glutamic acid)

Figure 8.2 Chemical structure of theanine.

8.4 Role of theanine in tea

Besides the 20 usual protein amino acids, it is known that hundreds of plants can synthesize unusual free amino acids, which are specific to one or other family or species: homoserine and *o*-acetylhomoserine in pea, canavanine in leguminous plants, 4-methylene glutamine in tulip, N^6-acetyllysine in beetroot, 3-carboxyphenylalanine in iris, *S*-methyl-L-cysteine in beans, etc. (Fowden, 1981). Many non-protein amino acids are considered to be the analogues of common amino acids due to their structural similarity. Possible roles for these often abundant non-protein amino acids in plants have been proposed, including defence against unfavourable environmental conditions, nitrogen storage and growth regulation (Wink, 1997; Morot-Gaudry *et al.*, 2001).

Theanine is a unique free amino acid first discovered in tea (*C. sinensis*). It is the analogue of glutamine. Theanine is found in every part of the tea bush except for the fruit and contributes the major part of free amino acids in tea (Selvendran & Selvendran, 1973). However, the physiological role of theanine in tea remains uncertain. It is possible that theanine is primarily involved in the storage and transport of nitrogen in a non-toxic form. Tea plants typically grow under acidic soil conditions and utilize ammonium as the main nitrogen source. Excess ammonium in cells is toxic to some species or families of plants and can cause symptoms such as chlorosis of leaves or suppression of growth (Britto & Kronzucker, 2002).

In Feldheim *et al.*'s study (1986), the content of theanine in tea seedlings was investigated. The level of theanine increased tenfold within the first 20 days and reached a maximum at 45 days after planting. After 45 days, the level of theanine decreased steadily and had reduced by about 80% at 90 days. They concluded that in the seedling, theanine might contribute to the regulation of growth, serving not only as the reservoir and transport form of nitrogen but also as the first step in the synthesis of skeletal carbon compounds. Kito *et al.* (1968) monitored the turnover of theanine in tea by investigating the distribution of the isotopically labelled *N*-ethyl carbon of theanine in tea seedlings and young shoots. They found that the *N*-ethyl carbon of theanine was significantly incorporated into the phloroglucinol nucleus of catechins. The incorporation was significantly affected by illumination. This finding was confirmed in young shoots from adult trees, which were fed with isotopically

labelled theanine. These results suggested that theanine might be one of the precursors of catechins, found at very high levels in tea. A study (Tanaka *et al.*, 2005) has demonstrated that theanine was degraded to a Strecker aldehyde and conjugated with polyphenol A rings to generate a novel polyphenol, ethylpyrrolidinonyl theasinensin, during production of black tea.

8.5 Metabolism of theanine in tea

Since the discovery of theanine by Sakato in 1949, theanine metabolism in tea has been studied on and off for 60 years. It has been pointed out that tea is normally grown in acidic soil and prefers ammonia over nitrate as an inorganic nitrogen source. The application of ammonia can increase theanine levels, as well as total nitrogen content, in tea. It has also been reported that shading can increase theanine levels. In 1965, Sasaoka suggested that tea contained a unique enzyme, which was capable of ligating glutamic acid and ethylamine to form theanine via a γ-glutamyl peptide bond. Takeo's study (1974) suggested that ethylamine, one of the theanine precursors, might be produced from L-alanine by decarboxylation. The other precursor, L-glutamic acid, was thought to be mainly generated by glutamine synthetase/glutamine:2-oxoglutarate aminotransferase (GS-GOGAT) following ammonium assimilated in the roots (Takeo, 1980). Several studies (e.g. Sasaoka *et al.*, 1965; Wickremasinghe & Perera, 1972) have reported that theanine is mainly synthesized in roots and transported to young shoots (see below), in which it is hydrolysed into glutamic acid and ethylamine by an enzyme called theanine hydrolase (Tsushida & Takeo, 1985). Whilst ethylamine may be converted into the phloroglucinol nucleus of catechins, the released glutamic acid from theanine would be expected to contribute to the biosynthesis of numerous physiologically important compounds such as proteins, chlorophyll, nucleotides and γ-amino butyric acid (GABA).

8.6 Theanine synthase

In 1963, Sasaoka demonstrated the presence of an enzyme in tea seedlings, which was capable of catalysing the synthesis of theanine from glutamate and ethylamine in the presence of ATP:

$$\text{ATP} + \text{L-glutamate} + \text{ethylamine} \rightarrow N(5)\text{-ethyl-L-glutamate} + \text{ADP} + \text{phosphate}$$

In two further studies (Sasaoka *et al.*, 1964a, 1964b), it was shown that enzyme extracts from pea seeds and pigeon liver could also catalyse theanine synthesis albeit under high pH conditions (~pH 8.5). However, the enzyme extract from tea seedling had its optimum pH at 7.5. The K_m value of the tea

seedling extract was shown to be much lower than those obtained with the pea seed and pigeon liver extracts. Furthermore, the theanine synthesis activity of the tea extract was not inhibited in the presence of ammonia, whereas the theanine synthesis activity of enzyme extracts from pea seeds and pigeon livers was. This suggested that there was a unique enzyme in tea, with a specific affinity for ethylamine. In 1965, Sasaoka further characterized the properties of theanine synthase (L-glutamate:ethylamine ligase). However, although the distinct activity of theanine synthase had been demonstrated, the purification of this enzyme has never been completed due to its lability. In Sasaoka's study, the preparation of the enzyme was carried out at 0.5°C, but even under these conditions, the loss of activity within 24 hours was 35%.

A patent by a Japanese company (Yukitaka *et al.*, 2006) claims that two isoforms of theanine synthase have been isolated from a tea cDNA library. The selected candidates were cloned in *Escherichia coli* and the proteins over-expressed. After purification, the two putative enzymes were tested for theanine synthesis activity using Sasaoka's method. Based on the results, they claimed that the putative enzymes were two distinct isoforms of theanine synthetase.

8.7 Theanine hydrolase

Remarkably, little is known about the metabolism of theanine in tea. Kito *et al.* (1968) reported that the ethylamine moiety of theanine was incorporated into catechins. This suggested that theanine might not be the end product and might be involved in other biosynthetic pathways. Tsushida and Takeo (1985) described an enzyme capable of hydrolysing L-theanine in tea leaves. In this study, theanine hydrolase and glutaminase activities were studied by measuring enzymatically released ethylamine or ammonia from L-theanine or glutamine. Both enzyme activities have pH optima at 8.5, whereas glutaminase has a pH optimum at 6.5. The theanine hydrolase was purified 4.6-fold by diethylaminoethyl (DEAE)-cellulose chromatography using a linear concentration gradient of KCl. Although 74% of the enzyme activity was recovered, theanine hydrolase activity could not be separated from glutaminase activity. Because the yields of both enzyme activities were different, the enzyme hydrolysing theanine was suggested to be a different enzyme from glutaminase. Theanine hydrolase activity was inhibited by acidic amino acids and stimulated by L-malic acid. This suggested that certain amino acid and organic acids might regulate theanine hydrolysis in tea leaves (Tsushida & Takeo, 1985). Although Tsushida's study has suggested the possibility of the existence of theanine hydrolase, this enzyme has not yet been purified successfully due to its lability. Moreover, it remains unclear whether theanine is hydrolysed by theanine hydrolase or other enzymes which can catalyse the hydrolysis of γ-peptide bonds in a more non-specific fashion. It has been reported that theanine can be hydrolysed by an enzyme extract from

Pseudomonas aeruginosa (Soda *et al.*, 1966) and glutaminase from rat kidneys (Unno *et al.*, 1999).

8.8 The site of synthesis and transport of theanine in tea

The site of theanine synthesis has not been conclusively determined. Sasoaka's study in 1965 has been quoted several times as evidence that theanine is only synthesized in roots. However, Sasoaka's study merely demonstrated that decotylized seedlings (roots) contained theanine synthase activity. Wickremasinghe and Perera (1972) reported that theanine was synthesized in roots following an inconclusive study in which $^{14}CO_2$ was fed to a mature leaf of a tea seedling and the pattern of label accumulation was recorded via autoradiography. This appeared to suggest that assimilated C (sugar) was initially translocated to the roots to provide the required skeleton for N assimilation and that the assimilated N (theanine) was subsequently translocated to the growing tips. However, in this study, the identity of the labelled compounds was not determined. The contribution of other parts of the tea plant to theanine synthesis has been largely ignored. Conversion of nitrogen and carbon into amino acids takes place in all parts of plants (Lea, 1993), and the biosynthesis of theanine in parts other than the root cannot be ruled out.

It has been shown that theanine is synthesized (*in vitro*) by both leaves and roots of tea explants when supplied with ^{14}C-labelled glutamic acid (Navin Sharma, personal communication). Free amino acids were separated by using TLC, and the amount of ^{14}C incorporated into theanine was counted; however, it is not known whether this activity would be inhibited by ammonia.

Once the amino acids are formed in roots, they are transported to the above-ground parts of the plant via the xylem sap. Amino acids from source tissues such as mature leaves may be loaded into the phloem and transported to sink tissues, where they are required for biosynthesis. As a major amino acid in tea, theanine has been reported to be mainly synthesized in roots and transported to young shoots. However, the mechanism of transport of theanine remains uncertain. In plants, it has been established that amino acids are transported from cells to cells by AA/H^+ symports (Li & Bush, 1990). Physiological studies have identified four types of transporters: two for neutral amino acids, one for acidic amino acids and a fourth for basic ones (Li & Bush, 1991). Genes encoding amino acid transporters have been classified into at least five gene families. The encoded transporters display different substrate specificities and affinities and have distinct subcellular locations. The ATF family (amino acid transporters family) and APC family (amino acid-polyamine-choline family) are two major superfamilies. The ATF family was first described in *Arabidopsis.* It contains six subclasses including the amino acid permeases (AAPs), the lysine/histidine transporters (LHTs), the proline transporters (ProTs), the aromatic and neutral amino acid transporters

(ANTs), the putative auxin transporters (AUXs) and the γ-aminobutyric acid transporters (GATs) (reviewed by Rentsch *et al.*, 2007). The second largest gene family of amino acid transporters is the APC family, with two subclasses identified to date. The first subclass contains the cationic amino acid transporters, which have high affinity for cationic amino acids and can transport both essential neutral amino acids and the basic amino acid lysine with moderate affinity (Rentsch *et al.*, 2007). The other subclass contains the L-type amino acid transporters (LATs), which were identified from mammalian systems (Verrey *et al.*, 2004). Little is known about the biochemical properties of LATs in plants.

Among the ATF superfamily of amino acid transporters, the AAP subfamily has been characterized in most detail (Rentsch *et al.*, 2007). Eight members of AAPs (AAP1–AAP8) from *Arabidopsis* have been studied using heterologous expression systems. All AAPs use a proton-coupled mechanism and are able to transport a wide spectrum of amino acids with low or high affinities depending on substrate properties such as charge, ionization state, α-carbon configuration and size and geometry of side chains (Fischer *et al.*, 2002). AAP3 and AAP5 are the only transporters to transport the basic amino acids efficiently, whilst aspartate is transported with physiologically relevant affinity only by AAP6 and AAP8 (Okumoto *et al.*, 2002). Theanine is a neutral amino acid and an analogue of glutamine; therefore, it might be expected to be transported by an AAP-mediating glutamine transport. *Arabidopsis* AAP4 and AAP5 transporters are able to support growth of a transport-deficient yeast mutant in media with theanine as the sole nitrogen source, suggesting that these transporters are able to transport theanine (Unilever, unpublished results). However, whether theanine can be transported by all AAPs or requires a specific transporter remains unclear because no tea transporters have been characterized to date.

8.9 Other enzymes capable of synthesizing theanine

A number of enzymes involved in theanine synthesis and turnover have been partially purified, and their biochemical properties have been investigated. However, it remains uncertain whether there is a unique enzyme in tea, which can synthesize or hydrolyse theanine. Some studies have shown that crude enzyme extracts from different species can catalyse theanine synthesis under high pH conditions. Recently, a number of artificial methods have been utilized to yield large quantities of theanine using different enzymes, which can generate γ-peptide bonds to form glutamyl compounds. The most commonly used enzymes for large-scale theanine production reported in the literature are glutamine synthetase, glutaminase and γ-glutamyltranspeptidase. Suzuki *et al.* (2002) describe an optimized artificial enzymatic method to yield theanine using bacterial γ-glutamyltranspeptidase (GGT). Initially, the GGT was found to prefer Gln over ethylamine as a glutamyl acceptor to form

glu-gln as a major product. After optimization, the yield of theanine was increased to 60% of the final product. Yamamoto *et al.* (2005) reported enzymatically producing theanine using a bacterial glutamine synthetase coupled with yeast fermentation as a source of energy. This method could not yield optimal amount of theanine, because of the low preference of the GS for ethylamine. However, the GS from *Pseudomonas taetrolens* Y-30 shows a high preference for ethylamine during theanine production. Tachiki *et al.* (1998) developed another enzymatic method for industrially producing theanine using glutaminase (defined as group B, with the characteristic of hydrolysis prior to transfer reaction with some acceptors) from *Pseudomonas nitroreducens*. They demonstrated the artificial synthesis of theanine under high substrate (glutamine and ethylamine) concentration and at an optimal pH of 11. Although these enzymes from different sources are used to synthesize theanine *in vitro*, the properties of the enzymes in tea for theanine synthesis/metabolism still remain uncertain.

It is interesting that amongst plants, only tea synthesizes theanine and that it makes so much. At present, there are a number of unknowns:

1. What is the principal N transport compound in tea?
2. What is the principal site of theanine synthesis (root, stem or leaf)?
3. Does tea synthesize theanine using (a) a common enzyme (e.g. glutamine synthase or γ-glutamyl transferase) via an uncommon mechanism (e.g. elevated cytosolic pH) or does it (b) possess a novel enzyme?

8.10 Nitrogen uptake and transport

It has been reported that heavy application of nitrogen fertilizer increases the theanine level in tea (Okano *et al.*, 1997), suggesting that theanine metabolism in tea is closely linked to nitrogen uptake and assimilation. The most common forms of nitrogen taken up by plants are nitrate (NO_3^-) and ammonium (NH_4^+). Plants have evolved special systems to cope with the diverse soil nitrogen conditions found in different regions. Although most plants can take up and utilize both forms of nitrogen, some plants do prefer one form of nitrogen over the other. Hayatsu and Kosuge (1993) reported that in tea fields the major form of mineral nitrogen was nitrate, because the supplied ammonium was rapidly converted to nitrate by nitrifying micro-organisms, at low pH. However, tea is reported to prefer ammonium over nitrate as the nitrogen source. It grows in acidic soil and absorbs ammonium more rapidly than nitrate (Ishigaki, 1978; Hoshina, 1985). Morita *et al.* (1998) demonstrated that, when tea was supplied with both forms of N at equimolar concentrations, the uptake of ammonium was twice that of nitrate. However, when the concentration of nitrate was much higher than that of ammonium (tenfold), the uptake of nitrate was twice as high as that of ammonium. They also found that the transport of nitrate to young leaves was much more rapid than that of

ammonium, and ammonium was largely retained in the roots or lower stems (Morita *et al.*, 1998). In practice, ammonium has become the major form of N fertilizer applied in the field, because the amount of nitrogen absorbed is higher when ammonium is applied than when nitrate is given (Ishigaki, 1978; Hoshina, 1985).

8.11 Nitrate transporters

Despite the importance of these two forms of nitrogen, their transport in tea at the molecular level remains unclear. Plants obtain nitrate from the soil by absorbing it across the plasma membrane (PM) of epidermal and cortical cells of the root. Physiological studies have demonstrated that the uptake of nitrate is an active transport process. The energy required is generated by the H^+-pumping activity of a PM H^+-ATPase. Kinetic studies have shown that roots contain three distinct nitrate transport systems. Two of them have high affinity for nitrate (HATS) and the other has low affinity (LATS). One of the high-affinity systems is highly induced by the external nitrate supply (iHATS), and the other is constitutively expressed (cHATS) (Aslam *et al.*, 1992). The regulation of nitrate uptake appears to be complex and to be negatively feedback-regulated by the products of nitrogen assimilation (Forde, 2000).

Molecular biological studies have identified two families of nitrate transporters. One is known as the NNP (nitrate-nitrite porter) family and the other as the PTR (peptide transporter) family. The transporters of both families are represented by multiple genes, regulated differentially. The transporters of the NNP family, encoded by *NRT2* genes, have high affinity for nitrate (HATS). Nitrate transporters of the PTR family, encoded by *NRT1* genes, are the components of low-affinity transport systems (LATS).

The NNP family of high-affinity nitrate and nitrite transporters occurs in both prokaryotes and eukaryotes and is one of 17 families of transporters belonging to the major facilitator superfamily (MFS). Proteins belonging to this superfamily are typically 500–600 amino acids in length and have two sets of six transmembrane helices connected by a cytosolic loop (Forde, 2000). The members of the NNP family can be further classified into three groups, based on their hydropathy profiles (for a review, see Forde, 2000). Plant members belong to the type III group, having an extended (70 amino acids) C-terminal domain. Presence or absence of an additional N-terminal sequence of approximately 20 amino acids allows further classification into type IIIb or IIIa, respectively. The N- and C-termini as well as the central loop are predicted to lie on the cytosolic side of the membrane. The function of these domains is uncertain, but based on similarities to other members of the MFS superfamily, it is predicted that they have some regulatory functions. For example, the C-terminus of an ATP-binding subunit of the ATP-binding cassette (ABC)-type nitrate/nitrite transporter of cyanobacterium *Synechococcus* PCC 7942

was shown to have a regulatory role in determining the sensitivity of the transporter to inhibition by NH_4^+ (Kobayashi *et al.*, 1997).

Eukaryotic NNPs are involved in nitrate and nitrite influx. The first member of the family to be cloned and properties investigated via heterologous expression was the *crnA* from *Aspergillus nidulans*. A mutation in this gene confers resistance to chlorate and a partial defect in nitrate uptake (Unkles *et al.*, 1995). Based on homology to crnA, putative *NRT2* genes (NNP family) have subsequently been cloned from a number of higher plant species (Forde, 2000). In *Arabidopsis*, seven *NRT2* genes have been discovered (*AtNRT2.1*∼*AtNRT2.7*). Comparison of intron–exon structures and protein domains, as well as phylogenetic studies, supports the hypothesis that the NRT2 members are distributed into two groups. The AtNRT2.1, 2.2, 2.3, 2.4 and 2.6 proteins share similar domain positions and features and belong to the first group. The second group contains AtNRT2.5 protein, which shares a common domain with yeast YNT1 protein, and AtNRT2.7 protein whose structure is identical to crnA (Orsel *et al.*, 2002).

In *Arabidopsis*, the differential expression pattern of the *NRT2* family has been studied (Orsel *et al.*, 2002). *AtNRT2.1*, *AtNRT2.4*, *AtNRT2.5* and *AtNRT2.6* are primarily expressed in roots, whilst the expression of *AtNRT2.7* is specific to aerial parts. *NRT2.1* transcript accumulates mainly in the epidermis and cortex of the mature root regions (Nazoa *et al.*, 2003) and expression is induced by nitrate, but repressed by high nitrogen status through negative feedback regulation, involving reduced nitrogen metabolites, such as ammonium or amino acids (Miller *et al.*, 2007). The expression of *NRT2* genes is thought to be regulated by feedback from nitrogen metabolites in many plant species (Touraine *et al.*, 2001). Some studies have suggested that some of the NRT2 family may require a second gene product for functional activity (Miller *et al.*, 2007). In the case of AtNRT2.1, a second protein, NAR2, is required to mediate nitrate transport (Orsel *et al.*, 2006). Mutations in this gene (*atnar2.1-1*) give a stronger phenotype (greater deficiency in HATS) than the *NRT2.1* mutants in *Arabidopsis*. This appears to be because the transporter protein is not correctly targeted to the plasma membrane, suggesting that the NAR2 proteins can facilitate targeting of some NRTs to the plasma membrane. Krouk *et al.* (2006) found that the expression pattern of *AtNAR2.1* almost exactly parallels that of *AtNRT2.1* and is similarly repressed by feedback regulation from nitrogen metabolites.

The PTR family is an unusual family of nitrate transporters, which also belongs to the MFS. In *Arabidopsis*, 53 members of NRT1 family have been identified (Miller *et al.*, 2007).

AtNRT1.1 (formally known as CHL1) was originally identified as a contributor to LATS (Tsay *et al.*, 1993). AtNRT1.1 is predicted to have two sets of six transmembrane domains, flanking a central hydrophilic region containing a large number of charged residues (Forde, 2000), a secondary structure remarkably similar to fungal high-affinity nitrate transporters (Forde, 2000). Expression of *AtNRT1.1* is highly tissue-specific at primary and lateral root

tips and in stomata, where it has a role in water stress responses (Guo *et al.*, 2003). Two reports have shown that AtNRT1.1 also has a role in nitrate uptake at low external nitrate concentrations (<500 μM), which means it may also act as a high-affinity system (Wang *et al.* 1998; Liu *et al.*, 1999). Evidence from oocyte expression studies demonstrated that AtNRT1.1 is active in both the high-affinity and low-affinity ranges. Together with *in vivo* analysis of the *chl1* mutants, it has been suggested that AtNRT1.1 is a dual-affinity transporter with two distinct K_ms for nitrate. Like *NRT2* genes, AtNRT1.1 is strongly induced by nitrate. However, the *AtNRT1.1* gene can also be induced in the absence of external nitrate, by a sudden reduction in the external pH (Tsay *et al.*, 1993). Unlike the *NRT2* gene products, which are down-regulated by the nitrogen assimilation metabolites, AtNRT1.1 expression is less susceptible to feedback repression.

The *AtNRT1.2* gene product, expressed in oocytes, lacks the high-affinity activity. It was shown to be constitutively expressed in root epidermal cells and has a K_m for nitrate of about 6 mM in oocytes (Huang *et al.*, 1999). There are many other candidate genes among the NRT1/PTR family. *AtNRT1.4* has a very specific pattern of expression in the leaf petiole where it has a role in nitrate accumulation within these tissues (Chiu *et al.*, 2004). Expression of *AtNRT1.3* was nitrate induced in the leaf, but repressed in the root and does not seem to be a significant contributor to LATS (Okamoto *et al.*, 2003; Miller *et al.*, 2007).

8.12 Ammonium transporters

Ammonium is an important source of nitrogen and its transport has been studied in many plants. Ammonium is generally taken up more rapidly than nitrate, when both forms of ionic nitrogen are provided at similar concentrations. This makes sense given the extra energy the plant must expend in reducing nitrate to ammonium before it can be incorporated into organic compounds (Howitt & Udvardi, 2000). Although ammonium, generally speaking, is taken up more rapidly than nitrate, it is not used for long-distance transport of nitrogen within the plant. Instead, most of the ammonium transported into plant cells is assimilated locally via glutamine synthetase in the cytoplasm and plastids (Howitt & Udvardi, 2000).

Much of the knowledge about ammonium transport in plants comes from physiological studies. Biphasic kinetic studies of ammonium uptake in several species of plants have indicated that there are at least two distinct transport systems: one has high-affinity and the other has low-affinity for ammonium. The high-affinity transport system (HATS) is regulated by the nitrogen status of the plant. In general, conditions of nitrogen deprivation lead to increases in the activity of HATS, while high concentrations of ammonium, or metabolites of its assimilation, lead to repression of HATS activity. The

low-affinity ammonium transport system (LATS) has a linear increase in activity in response to an increase in ammonium concentration.

Molecular biological studies have identified two families of ammonium transporters, namely, AMT1 and AMT2. The *AMT1* gene products in *Arabidopsis* were the first ammonium transporters in plants to be studied. Sequencing of the *Arabidopsis* genome allowed another five homologues of *AMT* genes to be identified (Gazzarrini *et al.*, 1999; Sohlenkamp *et al.*, 2000). Phylogenetic analysis revealed that AtAMT1.1–AtAMT1.5 share the highest homology to each other and clustered with cyanobacterial ammonium transporters, forming the AMT subfamily of ammonium transporters. AtAMT2 sequence is more closely related to the ammonium transporters found in *Saccharomyces cerevisiae* (Mep1 to 3) and in *E. coli* (AmtB), forming the MEP subfamily (Ludewig *et al.*, 2001; Loqué & von Wiren, 2004). Screening of a database from tomato (>150 000 expressed sequence tags, ESTs) identified three members of AMT1 and one putative member of AMT2 (Lauter *et al.*, 1996; von Wiren *et al.*, 2000). Interestingly, rice was reported to possess a larger number of ammonium transporter genes than *Arabidopsis* and tomato (Suenaga *et al.*, 2003; Sonoda *et al.*, 2003a, b). Phylogenetic analysis subdivided AMT-homologues from *Arabidopsis*, tomato and rice, into clades (Loqué & von Wiren, 2004).

The structure of several bacterial AMT family transporters suggests that the ancestral *AMT* gene may have had a classical 'six plus six' topology with intracellular N- and C-termini. However, many eukaryotic ammonium transporters appear to have lost the N-terminal helix and in some cases also either helix 8 or helix 9 (Howitt & Udvardi, 2000). The proposed transmembrane topology for AtAMT1.1 is ten transmembrane helices, with the N- and C-termini extracytoplasmic. In some plant ammonium transporters, the N-terminal hydrophobic region appears to have evolved into a localization signal. AtAMT1.1 has a weakly predicted signal sequence with the cleavage site between residues 20 and 21 (Nielsen *et al.*, 1997) which should place the mature N-terminus outside the cell.

Like the nitrate transporters, some *AMT1* genes are expressed in root hairs, suggesting that they have a role in uptake of ammonium from the soil (Lauter *et al.*, 1996; Ludewig *et al.*, 2002). However, in contrast to nitrate transporter genes, the expression of some *AMTs* is repressed by the presence of ammonium. In *Arabidopsis*, AtAMT1.1, 1.2 and 1.3 were all found to be highly expressed in roots, but expression of AtAMT1.1 and AtAMT1.2 was also found in shoots, being highest in mature leaves (Gazzarrini *et al.*, 1999). All three *AMT1* genes were also found to be highly responsive to N status and diurnal changes. Transcript levels of *AtAMT1.1* increased most when nitrogen was limiting. *AtAMT1.3* transcript levels peaked with ammonium uptake at the end of the light period, suggesting that this gene provides a link between nitrogen assimilation and carbon provision in roots (Gazzarrini *et al.*, 1999). Rawat *et al.* (1999) showed that *AtAMT1.1* gene expression negatively correlated with root glutamine concentrations, suggesting glutamine

acts as a metabolic trigger down-regulating the transcription of *AtAMT1.1*. High-affinity ammonium uptake decreased by only 30% in an AtAMT1 T-DNA insertion mutant, suggesting that other components of the AMT family may compensate for loss of AtAMT1.1 (Kaiser *et al.*, 2002). Furthermore, the double-insertion mutant, *atamt1.1-1:atamt1.3-1*, showed a decreased sensitivity to the toxic analogue methylammonium and a decrease in the ammonium influx of up to 70%, relative to wild-type plants, suggesting a contribution of both AtAMT1.1 and AtAMT1.3 to the overall ammonium uptake in *Arabidopsis* roots (Loqué *et al.*, 2006).

AtAMT2 proteins are most closely related to the putative ammonium transporters from bacteria. The isolated AtAMT2.1 cDNA from *Arabidopsis* roots was functionally expressed in a yeast mutant defective in all three transporter genes and restored the ability to uptake ammonia. This confirmed that AtAMT2.1 is a *bona fide* ammonium transporter (Sohlenkamp *et al.*, 2000). AtAMT2.1 has a topology similar to AtAMT1.1; therefore, the tertiary structure and molecular mechanism of AtAMT2.1 are likely similar to other plant AMT proteins, despite its divergent primary structure. The *AtAMT2.1* gene was found to be expressed in all organs of *Arabidopsis* and is subject to nitrogen regulation, at least in roots, where expression is partially repressed by high concentrations of ammonium nitrate and derepressed in the absence of external nitrogen (Sohlenkamp *et al.*, 2000).

AMT gene expression has been found to be differently regulated in other plants. In tomato, *LeAMT1.2* transcript levels in roots are up-regulated upon application of ammonium (von Wirén *et al.*, 2000). In rice, replacing ammonium with glutamine can still trigger the induction of *OsAMT1.1* and *OsAMT1.2* (Sonoda *et al.*, 2003b).

Although nitrate and ammonium transport in plants has been studied at both the physiological and molecular levels, the uptake and transport of both forms of nitrogen in tea plants are less well characterized. Further studies on how nitrate and ammonium are taken up and transported in tea and their regulation both at the physiological and molecular levels are important for a more complete understanding of theanine metabolism in tea.

8.13 Nitrogen assimilation by GS (glutamine synthetase) – GOGAT (glutamate synthase)

Theanine is thought to be made by the formation of a γ-glutamyl peptide bond between glutamate and ethylamine, catalysed by the enzyme theanine synthase (Sasaoka *et al.*, 1965). An understanding of the metabolism of glutamate and ethylamine, the two precursors of theanine, is therefore fundamental to an understanding of the synthesis of theanine. Takeo (1980) investigated two pathways related to amino acid synthesis in tea roots: GS-GOGAT and glutamate dehydrogenase. He found that L-methionine-DL-sulfoximine (MSO), an inhibitor of GS activity, suppressed the assimilation of ammonia

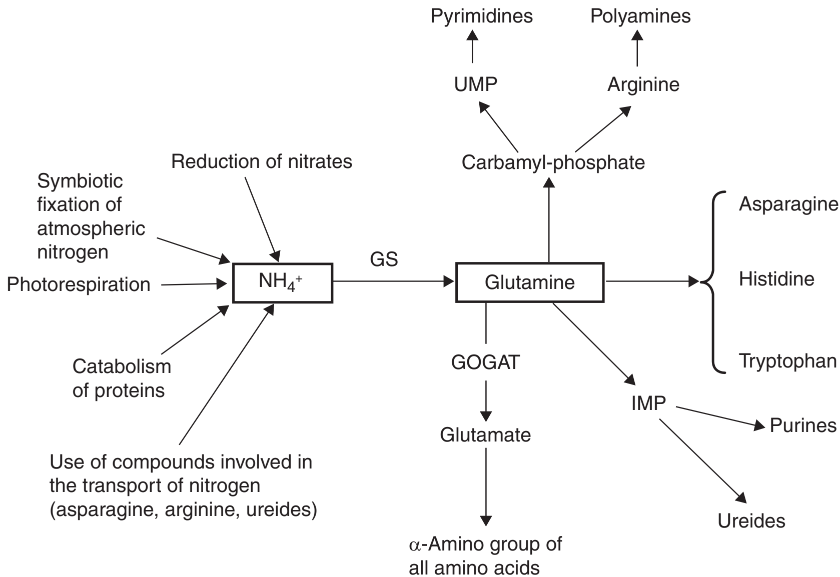


Figure 8.3 Metabolism of ammonium and glutamine in higher plants. IMP, inosine monophosphate; UMP, uridine monophosphate.

into amino acids and amides, especially theanine, and concluded that the GS-GOGAT system was the key entry point for assimilating ammonia in tea roots.

Since the discovery of glutamate synthase in 1974 (Lea & Miflin), it has been generally accepted that glutamine synthetase (GS; EC 6.3.1.2) and glutamate synthase (GOGAT; EC 1.4.7.1) are two key enzymes involved in the primary assimilation of ammonium in higher plants. The use of mutants deficient in glutamine synthetase, or glutamate synthase, or both, has confirmed their role in reutilization of photorespiratory-released ammonium in leaves (Lea *et al.*, 1989; Lea & Forde, 1994).

In plants, the ammonium ion is first incorporated into glutamate by a reaction catalysed by GS to form glutamine. The amide group of glutamine is then transferred by GOGAT to α-ketoglutarate to form two molecules of glutamate. One of the glutamate molecules is recycled to form glutamine, and the other is transferred to other biological pathways (Fig. 8.3).

The amide group of glutamine can also contribute to various nitrogenous compounds. In tea, glutamate molecules generated by GS-GOGAT are thought to be incorporated into theanine.

8.14 Biochemical properties of glutamine synthetase in plants

The structure, biochemical properties and regulation of GS have been studied extensively in plants. GS in higher plants has an estimated molecular weight

of 350–400 kDa and is assembled from eight identical subunits. The structure of GS has been investigated in soybean root nodules, yellow lupin root nodules, pea leaf, pumpkin root and leaf (Forde & Cullimore, 1989). Electron microscopic evidence from these studies has demonstrated that GS in plants consists of an octamer composed of two identical tetramers. A study of type II GS from *Phaseolus vulgaris*, using low-resolution electron microscopy, has demonstrated that it consists of an octamer, composed of two tetramers placed back-to-back and rotated 90° with respect to each other (Llorca *et al.*, 2006). An interesting finding from this study was the fact that the tetrameric ring is not built from four monomers, but rather from the interaction of two dimers, and hence each tetramer contains two active sites (one active site per dimer). This finding was in contrast to earlier models in which type II GS was described as an oligomer possessing a quaternary symmetry (Llorca *et al.*, 2006). Unno *et al.* (2006) reported the crystal structure of maize GS. The maize enzyme appears to be a decamer composed of two face-to-face pentameric rings of identical subunits, with a total of ten active sites, each formed between every two neighbouring subunits within each ring.

Early studies demonstrated that GS was widely distributed in plants and occurred in two major forms, one in the cytosol and the other in the chloroplast. These isoforms could be isolated by ion exchange chromatography. In leaves, GS is present in the chloroplast (GS2) and in the cytoplasm (GS1). However, the proportion of GS1 and GS2 in leaves varies, depending on the species, developmental stage and environmental conditions. It was found that in chlorotic leaves, GS1 dominates the GS activities whereas GS2 appears and becomes the predominant form when plants are transferred to light (Hirel *et al.*, 1992). Edwards *et al.* (1990) found that cytosolic GS is preferentially expressed in the vascular tissue of leaves. Subsequent studies also confirmed the location of GS1 in phloem and related vascular tissues (Tobin & Yamaya, 2001). In wheat, it was shown that GS1 was detected in the connections between the mestome sheath cells and the vascular cells (Kichey *et al.*, 2005). Many studies have identified that GS activities predominately exist in roots or root nodules of *P. vulgaris*, maize, *Arabidopsis thaliana*, soybean and *Pisum sativum* (for review, please see Brugière *et al*., 2001).

8.15 Gene families of glutamine synthetase

In many species, GS is encoded by a small multigene family. Plants typically have a single gene per haploid genome coding for GS2 (plastid GS) and many genes coding for GS1 (cytoplasmic GS) (Brugière *et al.*, 2001). In *P. vulgaris*, four genes coding for GS1 and one gene coding for GS2 subunits have been identified and designated as *gln-α*, *gln-β*, *gln-γ*, *gln-ε* and *gln-δ* (Brugière *et al.*, 2001). In *P. sativum*, three GS1 genes and one GS2 gene were identified (Walker & Coruzzi, 1989). In *A. thaliana*, early studies by Peterman and Goodman (1991) identified three genes coding for cytosolic GS in roots,

but expressed in different cells. Since the completion of the full *Arabidopsis* genome, it was found that there were five putative genes coding for cytosolic GS. The genes of cytosolic GS have also been studied and investigated in other plants, such as maize (Martin *et al.*, 2006), rice (Ishiyama *et al.*, 2004a, 2004b; Tabuchi *et al.*, 2005) and potato (Teixeira *et al.*, 2005).

8.16 Regulation of plant glutamine synthetase

The regulation of GS activity in plant cells has been described at several different levels: (1) transcription of the gene family; (2) processing and stability of the mRNA; (3) translation; (4) subcellular localization, processing or modification of the primary GS polypeptides; (5) assembly of the subunits of isoenzymes; (6) activity modulations of holoenzymes and (7) enzyme degradation (Forde & Cullimore, 1989). The regulation of GS begins with the transcription of GS genes. These are regulated at various levels, such as organ/tissue specificity, developmental stage and environmental response. To date, most work has considered the organs that have a specific role in terms of nitrogen metabolism, namely root nodules, roots and leaves. In legume plants, GS mRNA levels and GS activity are predominantly found in roots and root nodules and increase during nodule development (Brugière *et al.*, 2001). By fusing a GUS reporter gene to the promoter of the *P. vulgaris gln-β* gene that encodes a subunit of cytosolic GS, it was found that the expression of GUS was 20- to 140-fold higher in roots than in leaves (Brugière *et al.*, 2001). Also, several studies have reported nodule-specific expression of cytosolic GS in legumes such as *P. vulgaris* and alfalfa (Brugière *et al.*, 2001). However, in pea, the expression of cytosolic GS in roots is slightly different. In pea, three cytosolic GS isoforms from roots have been classified into two types (one comprising two nearly identical or 'twin' GS genes). The mRNA of all three GS genes was shown to accumulate coordinately during nodule development, but not exclusively in nodules. The twin GS genes were shown to be present in cotyledons of dry seeds and accumulated from 3 to 9 days after imbibition (Walker & Coruzzi, 1989). Early studies of cytosolic GS from *A. thaliana* suggested that the GS1 genes were preferentially expressed in roots and germinated seeds, relative to leaves (Peterman & Goodman, 1991). A study using real-time polymerase chain reaction (PCR) and Western blotting has demonstrated that, in roots, the mRNA and protein content of GS1 are more abundant than those of GS2, suggesting that GS1 is the predominant isoenzyme in roots (Ishiyama *et al.*, 2004a). In wheat, during the senescence of flag leaves, one isoform of GS1 (GS1b) was shown to be induced in the mesophyll cytosol whilst the other isoform (GS1a) remained constant, suggesting that GS1 may be involved in reutilizing of ammonium from protein hydrolysis (Kichey *et al.*, 2005).

It has been well documented that nitrogen supply can affect the regulation of GS genes and activities. Hirel *et al.* (1987) demonstrated that the addition

of ammonium to soybean roots, previously starved in nitrogen, leads to an increase in GS1 mRNA, suggesting that ammonium may induce the expression of cytosolic GS genes. Further investigations carried out by Hirel *et al.* (1992) have shown that ammonium, but not nitrate, increases the transcription of cytoplasmic GS in nodules of soybean. This finding was confirmed by experiments carried out in transgenic *Lotus corniculatus* (Miao *et al.*, 1991). In this experiment, the promoter of GS15 from soybean was fused with a GUS reporter gene. The chimeric gene was transferred into *L. corniculatus*, which was treated with external ammonia. Histochemical analysis revealed GUS activity mainly in roots and induced by ammonium (Fig. 8.4).

Another experiment carried out by Marsolier *et al.* (1993) showed that GS15 from soybean, previously found to be nodule-specific and ammonia-inducible, was also expressed in flowers and pulvinus of plants. The progressive 5′ deletion of the GS15 promoter, which was fused to a GUS reporter followed by transfer into *L. corniculatus*, identified the promoter elements necessary for ammonium stimulation as well as roots, nodules and pulvinus-specific expression.

In *Arabidopsis*, it has been shown that GS1 protein content increases with ammonium treatment, whereas the GS1 activity does not (Ishiyama *et al.*, 2004b). This discrepancy has been attributed to the kinetic properties and expression of four distinct isoenzymes, encoded by *GLN1.1*, *GLN1.2*, *GLN1.3*

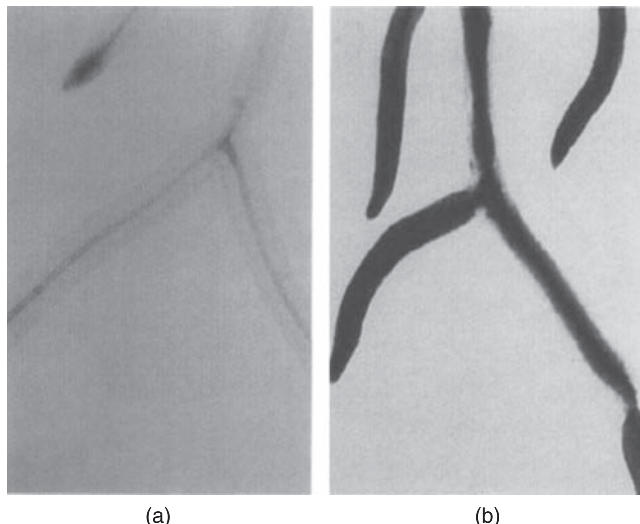


Figure 8.4 Histochemical localization of GUS activity in roots of *L. corniculatus* expressing a transgene corresponding to the translational fusion of a promoter of cytoplasmic GS of soybean with the GUS reporter gene. (a) Without ammonium and (b) with 10 mM $(NH_4)_2SO_4$ for 12 hours. Reproduced, with permission, from Miao *et al.* (1991).

and *GLN1.4* genes (Ishiyama *et al.*, 2004b). In this study, four genes of GS1 were shown to have different expression patterns under ammonium treatment. GLN1.2 (localized in the vasculature, low-affinity isoenzyme) was the only isoenzyme that was significantly up-regulated by ammonium, which correlated with the rapid increase in total GS1 protein. However, GLN1;1, GLN1;3 and GLN1;4 were repressed by ammonium or nitrogen metabolites at various levels. The expression of *GLN1*.3 (encoding a low-affinity isoenzyme) was not stimulated by ammonium, but the enzyme activity was significantly inhibited by a high concentration (80 mM) of glutamate. In contrast, the high-affinity isoenzyme, GLN1.1, accumulated in the surface layers of the roots when nitrogen was in limited supply and was down-regulated by an excess of ammonium. GLN1.4 was expressed in nitrogen-starved plants, but at a level tenfold lower than GLN1.1.

Light is another regulator of the expression of GS genes. In leaves of many plants, it has been shown that the chloroplastic GS genes are up-regulated by light. The regulation of GS2 genes and activities by light have been well studied in peas (Tingey *et al.*, 1988; Edwards & Coruzzi, 1989). In Edwards and Coruzzi's study (1989), it was shown that the mRNA for GS2 increased 20-fold after the geminated seeds were exposed to light for 72 hours. A four-fold increase was also observed in darkness, after exposure to a flash of red light, and the effect was reversible by far-red light. The gradual accumulation of GS2 mRNA induced by red light is similar to the mRNA accumulation pattern observed for phytochrome-sensitive genes, such as RUBISCO. Conversely, genes encoding cytosolic GS were not sensitive to light. Similarly, the regulation of leaf-specific GS by light in *Arabidopsis* showed that GS2 polypeptide levels increased during greening, whereas levels of GS1 polypeptides were unaffected, suggesting that GS2 is light-regulated (Peterman & Goodman, 1991).

8.17 Glutamate synthase (GOGAT) in plants

The second enzyme involved in the primary assimilation of ammonium is glutamate synthase (EC 1.4.1.13 and EC 1.4.7.1). It was first discovered by Tempest *et al* in 1970, who reported the presence of an enzyme in cell-free preparations of *Aerobacter aerogenes*. The original name was designated as glutamine:2-oxoglutarate aminotransferase (GOGAT). In plants, glutamate synthases, together with glutamine synthetase, were found to be the predominant system for nitrogen assimilation (Lea & Miflin, 1974). The GOGAT catalyses a glutamine-dependent reaction with 2-oxoglutarate to form two molecules of glutamate. It has been shown that there are two distinct types of GOGAT in plants. One is able to use ferrodoxin as the source of reductant, in a similar manner to nitrite reductase. The other is an nicotinamide adenine dinucleotide (NADH)-dependent form (Lea & Miflin, 1974; Lea & Ireland, 1999). Suzuki and Gadal (1982) were able to use antibodies to rice

Fd-glutamate synthase purified from green leaves to differentiate between the two distinct forms of glutamate synthase.

Fd-glutamate synthase can represent up to 1% of total proteins in leaves (Marquez *et al.*, 1988). In most plants studied so far, the Fd-glutamate synthase was shown to be monomeric, with a molecular mass ranging from 130 to 180 kDa (Brugière *et al.*, 2001; Lea & Miflin, 2003). However, in rice the enzyme was shown to have two subunits of 115 kDa (Lea & Miflin, 2003). The Fd-glutamate synthase from the cyanobacteria *Synechocystis* PCC6803, which is similar to the higher plant enzymes, was overexpressed in *E. coli*, and the reaction mechanism was studied in detail (Ravasio *et al.*, 2002).

The amidotransferase domain carries out the hydrolysis of glutamine to release the first molecule of glutamate and one molecule of ammonia, which is then transported through a channel to the flavin mononucleotide (FMN)-binding synthase domain. At the FMN-binding synthase domain, the released ammonia reacts with 2-oxoglutarate to form 2-iminoglutarate, which is then reduced by the co-factor FMN, to generate the second molecule of glutamate. Binding of both Fd and 2-oxoglutarate is required to produce a conformational change in the molecule that allows the glutamine to bind to the cysteine in the active site (Ravasio *et al.*, 2002; Lea & Miflin, 2003).

It has been shown that Fd-glutamate synthases are solely localized in the chloroplasts of leaves (Wallsgrove *et al.*, 1979) and algae (Cullimore & Sims, 1981). In tomato, Fd-glutamate synthase protein was found mainly in the bundle sheath chloroplasts (Botella *et al.*, 1988). In rice, Fd-glutamate synthase protein levels and activity were found to be highest in mesophyll cells, and found to a lesser extent in the parenchyma cells of the fully expanded green leaf blades. Levels were much lower in the leaf sheaths and developing non-green leaf blades (Yamaya *et al.*, 1992). In barley leaves, Fd-GOGAT was found to be present in the chloroplasts of mesophyll cells and vascular tissue (Tobin & Yamaya, 2001). Fd-GOGAT is also present in non-photosynthetic tissues and is localized in the plastids of roots. cDNA clones for Fd-glutamate synthase have been isolated from a number of species such as barley, maize and *Arabidopsis* (for review, see Lea & Miflin, 2003). In most plants studied so far, there is only one gene coding for Fd-GOGAT. In maize, the cDNA encodes a protein with a predicted sequence of 1616 amino acids. Further analysis of the maize and barley cDNA sequences in comparison with bacterial genes has identified regions corresponding to the glutamine-amidotransferase domain and the potential FMN-binding region (Lea & Miflin, 2003). However, Coschigano *et al.* (1998) suggested that in *Arabidopsis* there are two distinct genes coding for Fd-GOGAT (GLU1 and GLU2) with different expression patterns. Light was shown to be a major factor regulating the Fd-GOGAT activity in cotyledons and leaves in a range of plants (Ireland & Lea, 1999). In *Arabidopsis*, GLU1 mRNA is highly expressed in leaf tissue, and is specifically induced by light or sucrose. In contrast, the GLU2 mRNA is expressed at a lower level in leaves and preferentially accumulates in roots (Coschigano *et al.*, 1998). It has been suggested the Fd-glutamate synthase activity might

be induced by phytochrome and cryptochrome intermediates, via transcriptional activation of the corresponding genes (Zehnacker *et al.*, 1992).

The effect of externally supplied nitrogen on the regulation of Fd-GOGAT is species-specific and depends on factors such as the form of nitrogen supplied. In barley, a twofold higher level of Fd-glutamate synthase mRNA, protein and activity was detected in the leaves of plants grown in light on nitrate (Pajuelo *et al.*, 1997). In tobacco leaves, the levels of Fd-glutamate synthase mRNA, protein and activity were unaffected by a reduction in the availability of ammonium, either through the inhibition of nitrate reduction or the suppression of photorespiration (Migge *et al.*, 1997).

Most of the pyridine nucleotide-dependent glutamate synthases use NADH as the source of reductant (NADH-glutamate synthase; EC 1.4.1.14). However, NADPH-dependent forms have also been detected (Inokuchi *et al.*, 2002). NADH-GOGAT has been purified from rice suspension culture cells and root nodules and found to be monomeric, with a molecular mass of 190–200 kDa (Ireland & Lea, 1999). The structure of NADPH-GOGAT from *Azospirillum brasilense* has been studied in detail, indicating that the enzyme contains an α-subunit and a β-subunit (Binda *et al.*, 2000; Ravasio *et al.*, 2002). It has been proposed that the α-subunit contains two domains, one carrying out the glutamine amidotransferase reaction, and the other FMN synthase domain operating the transport of electrons to 2-iminoglutarate in a similar way to Fd-glutamate synthase (Lea & Miflin, 2003). In green leaves, the activity of NADH-GOGAT is low, in comparison to the Fd-glutamate synthase activity.

Yamaya *et al.* (1992) showed that NADH-GOGAT is localized in the large and small vascular bundles of the unexpanded rice leaves. In roots of N-depleted rice, NADH-GOGAT was found to be located in the central cylinder, apical meristem and secondary root primordial (Hayakawa *et al.*, 1999). Using specific antisera, the NADH-glutamate synthase protein was shown to be present throughout the *Rhizobium* symbiotic nitrogen-fixing zone of the nodule and also in the proximal region, which was not fixing nitrogen (Trepp *et al.*, 1999).

It has been shown that NADH-GOGAT is regulated by ammonium. The level of NADH-glutamate synthase protein and activity increased, at least tenfold, in roots within 1 day after transfer of nitrogen-starved seedlings to 1 mM ammonium chloride (Yamaya *et al.*, 1995). The accumulation of NADH-GOGAT mRNA was also observed when ammonium (50 μM) was applied to rice cell cultures or roots (Hirose *et al.*, 1997).

8.18 Glutamate dehydrogenase in plants

Glutamate dehydrogenase (GDH; EC 1.4.1.2) serves as a link between carbon and nitrogen metabolism, as it is capable of assimilating ammonia into glutamate or deaminating glutamate into 2-oxoglutarate and ammonia. It

has been found to be ubiquitous in living organisms (Stewart *et al.*, 1980). In higher plants, GDH activity has been found in many species, including maize, oats, wheat, barley (Miflin, 1970), peas, broad bean and lettuce (for review, see Stewart *et al.*, 1980). It has been well documented that two distinct GDH enzymes exist. One is a mitochondrial enzyme, which is generally referred to as NAD linked, and the other is a chloroplast enzyme, which is referred as NADP linked (Stewart *et al.*, 1980). The NADH-GDH isoenzymes are localized in the mitochondria, whereas NADPH-GDH isoenzymes are associated with chloroplasts. The number of distinct isoenzymes can vary in plant tissues during development and under different environmental conditions. The NADH-GDH activity has been found in a wide range of plant tissues including seeds, roots, hypocotyls, epicotyls, cotyledons and leaves (Stewart *et al.*, 1980). In angiosperm species, Lee and Stewart (1978) demonstrated that the enzyme activity of NADH-GDH is greater in roots than in shoots. NADPH-GDH has been found in shoot tissue (Leech & Kirk, 1968; Lea & Thurman, 1972) and in tobacco suspension culture cells (Washitani & Sato, 1977). NADH-GDH isoenzymes have a relative molecular mass ranging from 208 to 270 kDa, and are composed of six subunits of approximately 42–45 kDa. In *Arabidopsis*, two subunits (α and β) of NADH-GDH have been identified. Seven isoenzymes were demonstrated by native gel electrophoresis (Turano *et al.*, 1997). The slowest-migrating isoenzyme is a homohexamer of α-subunits, whereas the fastest-migrating isoenzyme is a homohexamer of β-subunits. The other isoforms of NADH-GDH are heterohexamers composed of different ratios of α- and β-subunits. The pattern of GDH isoenzymes varies in different plant organs, depending on environmental conditions.

Despite extensive knowledge of the physical and biochemical characteristics of GDH, the physiological role of GDH in plants still remains unclear. Prior to 1970, it was widely accepted that the main pathway of ammonia assimilation in living organisms was the reversible reductive amination of 2-oxoglutarate, catalysed by GDH. The evidence supporting this was based on ^{15}N-labelling studies in *Candida utilis* (Sims & Folkes, 1964) and *Chlorella* (Leech & Kirk, 1968). However, since the discovery of glutamate synthase by Lea and Miflin in 1974, the GS-GOGAT system has been regarded as the primary pathway of ammonia assimilation in plants (Miflin & Lea, 1980). Many studies have been undertaken to investigate the role of GDH in plants. Most of them have focused on whether GDH is involved in amination or deamination in plants.

Stewart *et al*. (1995) studied the role of GDH by comparing the metabolism of ^{15}N-glutamate in young seedlings of wild-type and GDH-null mutants of *Zea mays*. They demonstrated that in roots of wild-type plants, the major labelled products were glutamine and ammonium, whereas in GDH-null mutants, there was little or no labelling of glutamine or ammonium. In shoots of wild-type plants, N is recovered in the amide of glutamine, ammonium and amino groups of asparagine and other amino acids. In contrast, in the mutant, over 75% of the label is in the amino group of asparagine and little labelling

was found in glutamine. This suggested that the GDH in plants functions in the direction of oxidative deamination (Stewart *et al.*, 1995). By using *in vivo* NMR, *in vitro* GC-MS and automated $^{15}N/^{13}C$ mass spectrometry, Robinson *et al*. (1991) demonstrated that GDH is active in the oxidation of glutamate, but not in the reductive amination of 2-oxoglutarate.

Other studies strongly suggest that GDH acts as an alternative to the GS-GOGAT cycle for ammonia assimilation under high ammonium concentrations (Loyola-Vargas & de Jimenez, 1984; Yamaya *et al.*, 1984; Cammaerts & Jacobs, 1985; Melo-Oliveira *et al.*, 1996; Turano *et al.*, 1997). Loyola-Vargas and de Jimenez (1984) studied the role of GDH in nitrogen metabolism of maize tissues. They found that in roots, ammonium and glutamine increased NADH-GDH activity, whereas in leaves, the same metabolites inhibited GDH activity. Also, the amination and deamination reactions of GDH were shown to vary in tissues under different nitrogen conditions (Loyola-Vargas & de Jimenez, 1984). Melo-Oliveira *et al.* (1996) showed that whilst GDH1 mRNA accumulated to the highest level in dark-adapted or sucrose-starved plants, suggesting that under these conditions GDH1 may function in the direction of glutamate deamination, under conditions of carbon and ammonia excess, the GDH1 gene was found to be induced by exogenously supplied ammonium, suggesting that under certain conditions GDH1 may function in the direction of glutamate biosynthesis. Similarly, Tercé-Laforgue *et al.* (2004) demonstrated that in tobacco leaves, GDH remained at a low level during nitrogen starvation, but was highly induced when plants were grown on ammonium as a sole nitrogen source, a physiological situation during which leaf protein nitrogen remobilization is limited. They concluded that GDH did not play a direct role in nitrogen remobilization, but rather was induced by ammonium, supplied externally or released from protein hydrolysis during natural leaf senescence.

Lea and Miflin concluded that the most likely function of GDH is to act as a shunt to ensure that nitrogen metabolism does not excessively deplete 2-oxoglutarate levels in mitochondria and cells (Lea & Miflin, 2003). They suggested that GS, GOGAT and GDH all play complementary and non-redundant roles in both nitrogen metabolism and its interaction with carbon metabolism (Lea & Miflin, 2003). Figure 8.5 shows how GS, glutamate synthase and GDH may coordinate nitrogen metabolism in plants.

8.19 Regulation of theanine – genotypic factors

A number of studies have reported the occurrence of theanine in species of *Camellia* other than tea. An early study carried out by Tsushida and Takeo (1984) suggested that theanine was present in the seeds and seedlings of *Camellia japonica* and *Camellia sasanqua*.

Different varieties of *C. sinensis* have been found to have different levels of theanine (Navin Sharma, unpublished observation). Furthermore, a high

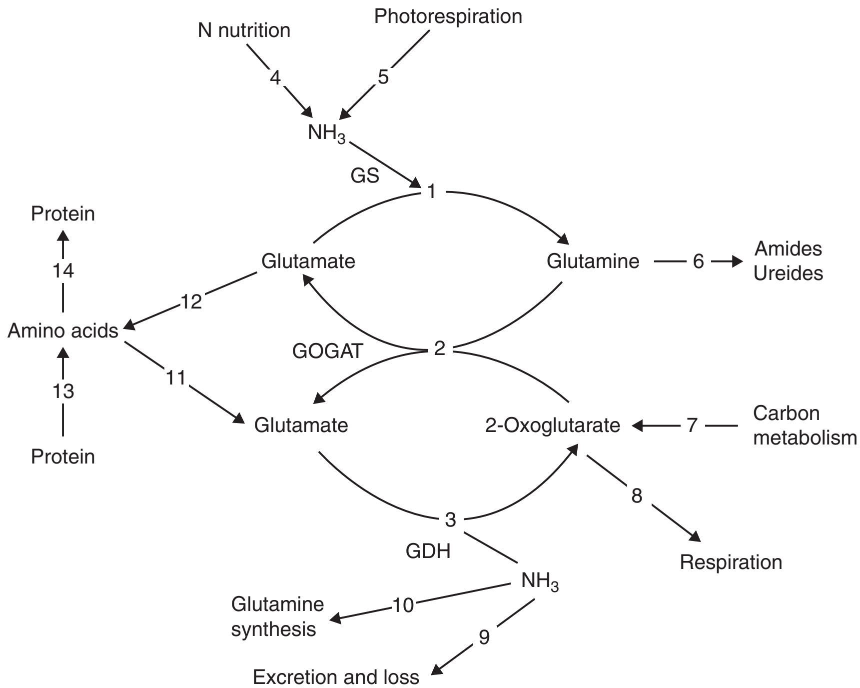


Figure 8.5 The role of GS, GOGAT and GDH in nitrogen metabolism and its interaction with carbon metabolism under different metabolic situations. Reproduced, with permission, from Lea and Miflin (1972).

Metabolic situation	Major reactions involved
Healthy, growing leaves in light	1,2,4,5,7,12,14
Germinating legume cotyledons	13,11,4,10,1,6
Carbon-starved non-green cells	13,11,3,8,9
Senescing leaf remobilizing nitrogen	13,11,3,1
Developing seeds	7,3,12,14
Excess nitrogen, stress, carbon limitation	13,11,3,8,9

theanine mutant has been identified by Professor Jian'an Huang at Hunan Agricultural University, China. This mutant, with white leaves, was found to contain two to three times of the normal theanine level.

8.20 Regulation of theanine – agronomic factors

There have been a number of reports in the literature that agronomic factors can affect theanine levels. Much of this work has focused on nitrogen

application, such as the level and form of nitrogen applied. Tea plants in Japan are typically supplied with a large amount of fertilizer nitrogen almost every month throughout the growth period (Tachibana *et al.*, 1996). Ammonium is the preferred form of nitrogen fertilizer applied in tea fields because the nitrogen is absorbed more efficiently when ammonium is applied than when nitrate is applied (Ishigaki, 1978; Hoshina, 1985). Ruan *et al.* (2007) also demonstrated that the concentration of free amino acids in shoots and roots, especially theanine and glutamine, was higher in NH_4^+ than in NO_3^- fed fields, suggesting ammonium was the preferred nitrogen form in tea fields. Okano *et al.* (1997) reported that the application of 6–12 N plots (1 N plot corresponding to 10 kg N/10 acres/year) increased theanine levels in shoots and favoured plant growth. The critical level for leaf quality was estimated to be about 12 N plots (Okano *et al.*, 1997). Our own studies have shown that, where tea is grown under poor N conditions, up to a five-fold increase in theanine levels in the whole shoot could be achieved by applying 600 kg N/ha. There are a few other agronomic factors that can influence theanine levels, such as temperature, illumination and plant growth regulators. Kito *et al.* (1968) reported that the amide group of theanine was incorporated into the phloroglucinol nucleus of catechin, which was controlled by light. Matsuura and Kakuda (1990) demonstrated that theanine accumulated in callus culture in the dark and that the optimum temperature for theanine accumulation was 25°C, suggesting that temperature and illumination affect theanine levels. It has also been reported (Takihara-Matsuura *et al.*, 1994) that the combination of plant growth regulators, benzyladenine and indo-3-butyric acid induced both growth and theanine accumulation in tea callus, whereas 2,4-dichlorophenoxyacetic acid functioned as a strong inhibitor. Also, in this study, it was found that increasing the concentration of sucrose or glucose in callus culture increased callus fresh weight, but the highest theanine accumulation was found in callus culture with a sucrose concentration of 30 g/L, suggesting that the suitable carbon source for theanine formation is sucrose and its optimum concentration is 30 g/L. These studies suggest that carbon metabolism and plant hormones are involved in the regulation of theanine levels in tea.

Drawing evidence from recent studies, a mechanism of theanine metabolism has been proposed (see Fig. 8.6).

8.21 Summary

The key enzymes involved in theanine metabolism in tea are thought to be theanine synthase and theanine hydrolase. The direct route to study the properties of these two enzymes would be to purify the enzymes from tea and obtain protein sequence information, which would allow us to study their expression patterns and biochemical properties. The purification of theanine synthase was first attempted by Sasaoka in 1965. However, it could only

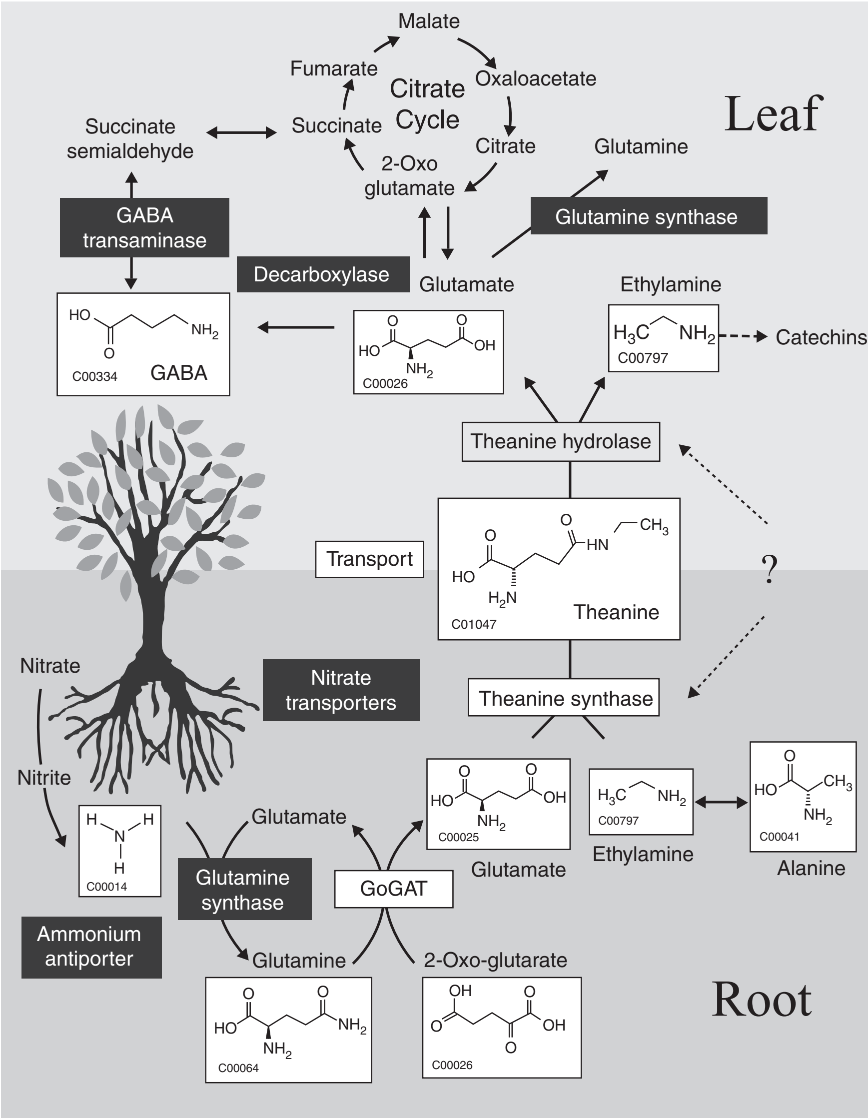


Figure 8.6 Proposed mechanism of theanine metabolism in tea. After ammonium is taken up, it is assimilated via the GS-GOGAT system to generate two molecules of glutamate. Theanine is synthesized by joining glutamate with ethylamine, catalysed by a novel enzyme, theanine synthetase (Sasaoka *et al.*, 1965). The site of theanine synthesis is not fully understood. Originally, it was reported that theanine was synthesized mainly in roots, but it is also possible that some theanine is synthesized in the leaves, where at least *in vitro*, theanine synthesis has been demonstrated. After theanine is transported to young shoots, it is hydrolysed to generate glutamate and ethylamine, by theanine hydrolase. The released glutamate from theanine is a source of N fuelling plant growth as well as the biosynthesis of functional molecules such as GABA. The released ethylamine from theanine may contribute to catechin metabolism in tea.

be partially purified because the enzyme is unstable. Theanine hydrolase has also been partially purified by Tsushida and Takeo (1984), but the activity was difficult to separate from glutaminase. A patent from Taiyo Kagaku Co. Ltd claims the protein and cDNA sequence of two isoforms of theanine synthase from tea (Yukitaka *et al*., 2006).

In our laboratory, two approaches were adopted to try to identify genes putatively involved in the synthesis and turnover of theanine. The first was to use bioinformatics to identify potential theanine synthase and hydrolase genes in our in-house tea cDNA collections. This approach was based on an assumption that theanine synthase has likely evolved from, and should therefore be structurally related to, an ancestral gene capable of catalysing a similar biochemical reaction, for example glutamine synthetase. The second approach was to identify genes whose expression was coordinately regulated with theanine levels, which are therefore likely to be involved in regulating theanine accumulation in the shoot (via metabolism, transport or regulation).

In the first approach, two putative genes (contig 928 and contig 1782) identified from our fresh and withered tea leaf cDNA library exhibited high homology to glutamine synthetase and γ-glutamyl hydrolase genes in the public databases. These enzymes are capable of forming/hydrolysing a γ-glutamyl peptide bond, which is the reaction needed to synthesize/metabolize theanine. Contig 928 encodes a full-length glutamine synthetase gene. Contig 1782 encodes a γ-glutamyl hydrolase homologue. This gene is intriguing because the encoded polypeptide appears to have acquired a potential chloroplast transit peptide (mis-targeting being a common feature of neofunctionalization). Both of these genes were cloned into the fungi *Pichia pastoris*, and overexpressed. Both proteins were partially purified using nickel-chelating affinity chromatography and their functions were characterized using both spectrophotometric and fluorescent high-performance liquid chromatography (HPLC). The results suggested that contig 928 may act as a glutamine synthetase, whilst contig 1782 may encode a putative theanine hydrolase.

In the second approach, tea shoots showing a fourfold variation in the theanine level were harvested from an experimental plot in Kenya. This variation was achieved by varying the time of collection (dawn vs dusk) and the nitrogen application (0 kg/ha vs 600 kg/ha). Global gene expression analysis was carried out using (i) a pan genome cDNA-AFLP analysis and (ii) a partial tea microarray to identify novel genes correlating with theanine levels in tea.

cDNA-AFLP is a gene expression tool that can theoretically achieve up to 70% genome coverage with no prior sequence knowledge. Quantitative temporal accumulation patterns of more than 14 000 tea transcript tags were determined and analysed. Over 2000 differentially expressed tea transcript tags were identified and 107 of these genes were sequenced. Importantly, five particularly interesting genes, which are likely involved in glutamate, and thus potentially theanine metabolism were identified, that is, glutamine synthetase 1.1 (CS001), glutamate synthase (AK151), NADH-glutamate

dehydrogenase (AK090), glutamine amidotransferase (AK114) and nitrate reductase (AK188).

The tea gene tags identified by cDNA-AFLP were added to our in-house tea cDNA collections, long and oligonucleotide probes designed, synthesized and used to construct a 3 K tea array. Microarray analysis was performed, and genes were identified according to fold differences in expression (scatter plot and ven diagram analysis) and expression patterns of interest (cluster analysis). A total of 101 genes were found to be up-regulated (at least 1.5-fold) only under condition NK4 (dawn and +N) that produced the highest level of theanine. Amongst these were a serine carboxypeptidase and a serine glyoxylate aminotransferase that warrant further investigation as potential theanine synthase genes. A total of 17 genes were found to be up-regulated (at least 1.5-fold) in both NK3 (dusk, +N) and NK4 (dawn, +N). Amongst these genes, nitrate reductase is particularly interesting.

Combining cDNA-AFLP analysis, qRT-PCR and tea microarray analysis, the global gene expression study identified a number of genes, which are potentially involved in nitrogen metabolism and appeared to correlate with theanine accumulation in tea. Having identified a number of candidate genes believed to be involved in theanine accumulation, the next step was to identify pragmatic pre- or post-harvest treatments that could be used to modulate gene expression (synthesis, turnover, transport and regulation); enzyme activity (synthesis, turnover); or biochemical interactions, with a view to enhancing theanine levels in shoot. A possible example is to use sugars or cytokine to increase nitrate reductase expression. Alternatively, approaches might be sought to reduce the expression (activity) of genes (enzymes) that exhibited an inverse correlation with theanine accumulation.

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Chapter 9

LEGUME NITROGEN FIXATION AND SOIL ABIOTIC STRESS: FROM PHYSIOLOGY TO GENOMICS AND BEYOND

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Abstract: Legumes are important components of the nitrogen cycle on land. Agricultural systems have traditionally relied much on legumes for nitrogen input because many species are able to establish symbioses with diazotrophic bacteria (rhizobia) and thus trade metabolites and reduced compounds. Photosynthates produced in the leaves are allocated to the root nodule to supply the bacteroids with carbon, in exchange for reduced nitrogen (ammonia) produced by the rhizobia from atmospheric nitrogen. Despite its major significance to plant breeding and sustainable agriculture, the impact of abiotic stresses on nodule development and stability and on symbiotic nitrogen fixation remains poorly understood, particularly at the molecular level. However, the study of model legume species and the development of a plethora of resources, particularly the elucidation of the genome sequences of three legume species, are now revealing many traits of agricultural importance in legumes as well as other aspects that are not easily studied in other plant models, such as *Arabidopsis* or rice. In this chapter, we will discuss the effects of abiotic stresses, such as drought, phosphate deficiency and aluminium toxicity, on symbiotic nitrogen fixation and provide perspectives on molecular approaches to the analysis of stress responses in legumes.

Keywords: aluminium; drought; nodule; phosphorus; rhizobia; symbiosis

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9.1 Introduction

It is ironic that one of the most limiting nutrients for crop production worldwide is nitrogen (N), and yet the air in earth's atmosphere is largely (78%) nitrogen gas (N_2). However, N_2 is very stable and relatively few prokaryotic organisms (called diazotrophs) have the enzymatic machinery to break the strong triple bond between the two N atoms and make them reactive with other atoms, such as hydrogen, and form ammonia (NH_3). This reaction is exothermic ($\Delta H = -92.4$ kJ/mol when using a catalyst) and has to be carried out under anoxic conditions, since nitrogenase is irreversibly inactivated by oxygen (O_2).

9.1.1 Nitrogen in the big picture

Out of the agricultural and industrial contexts, though, figures are massive: the oceanic fixation is estimated to account for over 400 Tg N per annum (Codispoti, 2007). And, indeed, N_2 fixation was found to occur even at extreme conditions, such as 92°C hydrothermal vents in the deep sea floor by the archaeal *Methanocaldococcus jannaschii* (Mehta & Baross, 2006).

The gaseous N can also be industrially fixed to NH_3, through the so-called Haber-Bosch process. This process was fundamental for the success of the Green Revolution, in which it guaranteed N inputs into crop systems for food production as well as industrial uses. Today, the Haber-Bosch process is responsible for 65% of the N fixation on the earth's lands (Conley *et al.*, 2009), consuming more than 1% of the world's energy supply (Smith, 2002). The fertilizer produced by this process contributes to sustain approximately 40% of the global population (Fryzuk, 2004), although at a price.

However, together with phosphorus, N is a major pollutant in eutrophied regions, with impacts on freshwater deposits and aquatic life, as well as the terrestrial ecological disequilibrium through disturbance of the food webs. Indiscriminate N application leads to imbalances in the N biogeochemical cycle of local ecosystems, creating potential ecological problems (Galloway *et al.*, 2008), since much of the applied N is readily lost. As in the case of biofuel crop in Brazilian fields, only 31% of N fertilizer applied as urea in sugarcane is assimilated by the plants (Trivelin *et al.*, 2002). Other studies corroborate that urea use efficiency is usually below 50% in field conditions for other crops (Choudhury & Khanif, 2001; Halvorson *et al.*, 2002). Crops are also responsible for N relocation: one metric ton of dry biomass (seeds and straw) contains 26–28 kg N for wheat, 16–17 kg N for rice, 9–11 kg N for maize and 7 kg N for sugarcane (compiled by Kennedy *et al.*, 2004). After harvest, a major part of this organic N is mineralized and returns to the cycling system.

Current losses of anthropogenic N from land to sea are estimated to be 48 Tg/year (Schlesinger, 2009), being approximately 1 Tg in the form of nitrous oxide (N_2O; Duce *et al.*, 2008), with grave consequences for the ozone layer and greenhouse effect, since this gas is 300 times more effective than

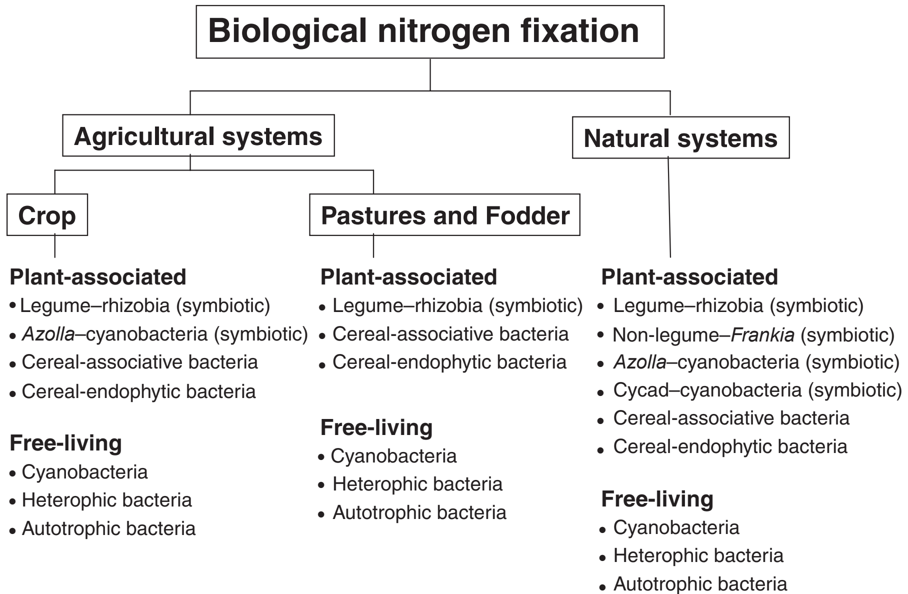


Figure 9.1 Biological elements of the nitrogen cycle. Reproduced from Herridge *et al*. (2008), with permission from Springer Science and Business Media B.V.

CO_2 in this regard. Atmospheric emissions of NO (also derived from fossil fuel combustion), NH_3 (volatilized mainly from fertilization and organic decomposition) and nitrate (NO_3^-) leaching to underground water are also major points of environmental concern related to N.

Several symbiotic nitrogen fixation (SNF) systems play important roles on the earth (Fig. 9.1) with great impact on agricultural production, as some non-legume associations: for example, the plant–fern–cyanobacterium tripartite symbiosis of rice, *Azolla* and *Anabaena azolla*; sugarcane/other grasses and endophytic bacteria as well as free-living soil diazotrophic bacteria (such as *Azotobacter*, *Acetobacter*, *Azospirillum*, *Anabaena*, *Clostridium* and *Nostoc*); alder (Betulaceae, *Alnus* spp.) and the actinorhizal bacteria *Frankia*. All in all, it is estimated that non-legume symbiotic associations may totalize roughly 23 Tg N biologically fixed yearly (Herridge *et al*., 2008). The tripartite symbiosis system of rice has been reported to be self-sufficient in 75% of the N requirements for the crop (Havlin *et al*., 2005).

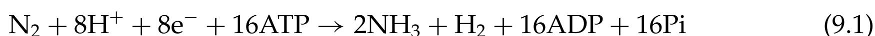
In agricultural fields, however, legumes are considered the main natural contributors for usable N inputs for its symbiotic association with rhizobial bacteria. In 2005, SNF in crop systems was estimated 40 Tg N, whereas the Haber-Bosch process fixed 121 Tg N, and natural ecosystems contributed with only 26 Tg N (Conley *et al*., 2009). Another estimative accounts SNF in the range between 50 and 70 Tg N annually, when also considering uncultivated grazing areas and non-legume N fixation symbioses (Herridge *et al*., 2008).

A more rational use of N in agricultural systems is possible with more efficient N application management, as well as the use of other N sources with rather gradual release (such as during organic matter decomposition) and the inoculation of non-symbiotic soil diazotrophs. Policies focusing on increasing N use efficiency are being strongly called upon, and the utilization of legumes in sustainable crop systems will be of help in this process. For this, understanding how root nodulation develops and how N fixation occurs symbiotically and improving N_2 fixation rates in legumes and free-living micro-organisms could direct to changes in the N fertilization paradigm used today in modern agriculture.

9.1.2 Legumes and nitrogen

In order to have reduced N as NH_3, legumes establish mutualistic interactions with specific diazotroph soil bacteria (*Rhizobium* spp.) and create the required conditions for N_2 fixation. Biologically fixed N is regarded as more environmentally friendly than the utilization of fertilizers produced by industrial fixation because the NH_3 generated in this process is readily assimilated into organic forms by the plant, only released gradually back to the environment during organic matter decomposition by bacterial nitrification to NH_3 and further denitrification to N_2.

Nitrogen derived from bacterial fixation is not free of charge: the plant trades it for reduced carbon that is used to sustain bacterial physiology as well as to produce the 16 adenosine triphosphates (ATPs) required by nitrogenase for N_2 reduction (Equation 9.1). Similarly to other plants, legumes are capable of taking N up from the soil as NO_3^- via specific (high- and low-affinity) membrane transporters located in the epidermal and cortex root cells (although shoot tissues are also capable of assimilating NO_3^-), and nitrate can be accumulated in the vacuole, or loaded to the xylem and sent to the shoot. The reduction of NO_3^- to nitrite (NO_2^-) and then immediately to NH_3 (since NO_2^- is highly toxic) utilizes indirectly 15 ATPs in these reactions, consuming up to 20% of the energy generated by root respiration (Bloom *et al.*, 1992):



$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi \qquad (9.1)$$

Thus, each symbiotically fixed NH_3 utilizes 8 ATPs for the reaction, but one should not be misled by the simplicity of the reaction stoichiometry when comparing SNF and NO_3^- uptake, since other factors must be included in the audit: the energy costs of development or expansion of specialized organs, cell differentiation, membrane polarization to sustain transporter systems, as well as transcription, translation, breakdown and activity regulation of gene products required for the process. A more global way to measure the total energy requirements involved is through quantifying carbon (C) expenditure. The cost of carbohydrate to N fixed was estimated to be 17:1 for several amide-exporting legumes (Witty *et al.*, 1983) and around 12:1 for soybean (Rainbird

et al., 1984), a ureide-exporting legume. There is much debate about the actual figures due to methodological problems of measuring C economy; the cost of symbiotic N_2 reduction in legumes was estimated to be between 2 and 3 mg C per mg N fixed, varying according to the species (Schulze *et al.*, 1999) and probably genotypes (Moreau *et al.*, 2008). SNF is assumed to require significantly more energy per N fixed than NO_3^- uptake and reduction. Indeed, early experiments with lupin (Pate *et al.*, 1979) show that SNF consumes 10.2 mg C/mg N fixed and NO_3^- requires 8.1 mg C/mg N whereas results for soybean followed the same trend (8.28 mg C/mg N and 4.99 mg C/mg N, respectively; Finke *et al.*, 1982). Data gathered by Kaschuk *et al.* (2009) show that N_2 symbiosis allocates 11–14% of photosynthesized C. The great evolutionary advantage of N_2-fixing legume species is, nonetheless, to easily thrive in low N environments where other species hardly strive.

Legumes arose on the earth approximately 60 million years ago (in the Paleocene, after the K/T extinction and concomitant with a major radiation of mammal, birds pollinating insects and angiosperms), and soon after approximately 58 million years ago (Lavin *et al.*, 2005; Sprent, 2007, 2008), some legume species evolved a novel organ in the root system to accommodate N_2-fixing bacteria and established a deal where the plant provides reduced carbon in exchange for reduced N (in the form of NH_3) under a well-controlled mechanism to avoid bacteria to overproliferate and become parasitic (i.e. pathogenic). In this regulatory mechanism (called 'autoregulation of nodulation'), still uncharacterized molecularly, two major components are involved: one signal translocated from the shoot (and dependent on the shoot size) and another originated in the root which the NO_3^- status plays a conspicuous regulatory role (Caetano-Anollés & Gresshoff, 1991; Oka-Kira & Kawaguchi, 2006; Okamoto *et al.*, 2009).

To trigger SNF in legumes, particularly *Rhizobium* species, which are closely related to *Agrobacterium*, establish a chemical talk with plant roots, which enclose the bacteria by endocytosis, originating novel organelles (symbiosomes) and, in some cases, leading to the development of novel organ structures (nodules) that create a hypoxic environment proper for nitrogenase activity. The enclosed bacterium suffers physiological and morphological changes which will enable their apparatus to fix N_2 (the N_2-fixing form enclosed in the symbiosome is then called 'bacteroid'). Bacteroids are then fed by the plant with reduced C (usually intermediates of the tricarboxylic acid (TCA) cycle, such as malate and succinate) in exchange for reduced N in the form of NH_3 that is exported to the plant cell cytoplasm, assimilated into organic forms and translocated to other parts of the plant.

There are two main types of nodule structures depending on the legume species: one showing determinate growth (which grows up to maturity, stops the development and starts N_2 fixation), such as in soybean, common beans and the model *Lotus japonicus*; and indeterminate growth (which holds a meristematic zone that guarantees a continuous growth of the organ concomitantly with N_2 fixation), such as in *Medicago truncatula*, alfalfa and pea.

Determinate nodules are homogenous in the inner tissues, presenting a parenchymatic core of infected cells with homogenous ontogeny (this trait evolved independently a few times during legume evolution and is considered more advanced than the alternative), whereas indeterminate nodules (present in most of the symbiotic legume species) display a more complex structure divided into zone I, the meristematic region located in the apex of the nodule; zone II, infection zone, where infection threads containing bacteria are engulfed by the plant cells, generating the symbiosome; interzone II–III, where the bacteria suffer a physiological transformation to bacteroids, the N_2-fixing form; zone III, where most of the N_2 fixation occurs; and zone IV, the senescence zone. The indeterminate nodule continues to grow, and the fixation zone today will be a senescence zone days later and so on, whereas in the determinate nodule, the core cells of the nodule develop simultaneously and the whole nodule dies afterwards (van de Velde *et al.*, 2006). Approximately 50 million years ago, legumes diverged in two clades, tropical and temperate, differing mainly in the type of nitrogenous compound translocated to the shoots (ureides and amides, respectively), but both clades contain examples of indeterminate and determinate nodule development.

It is also important to consider in the context of N uptake and fixation that the soil NH_3 (or its protonated form – ammonium, NH_4^+) can be directly taken up by the roots through specific transporters (possibly aquaporins and channels), but in opposition to NO_3^-, NH_3 cannot be stored in the cells for its toxicity, demanding a quick assimilation into organic forms before translocation to sink organs. The requirement of immediate NH_3 assimilation may compete for carbon skeletons with other biochemical pathways under certain situations, making it unfavoured in many species, despite its energetic advantage when compared to NO_3^- uptake; since its N is already reduced, ammonia assimilation consumes only 10% of root respiration (Bloom *et al.*, 1992; Schilling *et al.*, 2006). Organic N – such as amino acids, peptides and urea – can also be taken up by the roots via specific membrane transporters that are only now being discovered and characterized in plant species (Lee *et al.*, 2007; Rentsch *et al.*, 2007; Komarova *et al.*, 2008; Nasholm *et al.*, 2009).

9.1.3 Abiotic stresses in legumes

Legumes can rely solely in symbiotically fixed N for their physiological requirements, but since this reduced N is energetically more expensive than the soil N, external supply of reduced N, such as NO_3^-, boosts plant development (Moreau *et al.*, 2008). Yet, symbiotically fixed N accounts for 40–80% of the assimilated N under real agronomical conditions worldwide (compiled by Herridge *et al.*, 2008), with perennial legumes fixing about 110–220 kg N/ha per year, and annual legume crops, roughly half of it (Havlin *et al.*, 2005). Factors such as climate, soil properties (pH, physical properties and mineral availability, including N availability), soil biology (bacteria genetics, inoculum amounts in the soil), crop management, ecological relationships

and several other stresses impose restrictions to optimal N fixation. This fact, sometimes overlooked in basic studies of SNF, is fundamental to understand how it occurs under stress so that parameters could be adjusted for maximization of the process, which might translate to higher crop yields.

Abiotic stresses account to major, more than 50% of crop, losses worldwide (Bray *et al.*, 2000), where drought, salinity, aluminium toxicity and nutrient deficiencies (especially N and P) are the major components. Drought alone reduces soybean yields in approximately 40% (Specht *et al.*, 1999). In acidic soils, which are prevalent in tropical regions of the globe, Al and P are major limitations to crop production. Here is another irony of nature – phosphate and water are plentiful on the planet, however not accessible for crop systems. Phosphate is generally unavailable in tropical, weathered soils, where it is strongly adsorbed onto the clay particles and mostly unavailable for root uptake. Saltwater accounts for 97% of the water on the planet, and rainfall over land is distributed unevenly (space- and time-wise), not always available when the crops most need it to maximize yields. Intensive irrigation is costly and may lead to soil salinization. Nevertheless, in natural situations, plants evolve mechanisms to cope with such stresses, but in an agricultural context, they hinder crop yields and hamper the achievement of their genetic potentials.

By understanding how abiotic stresses affect plant development and crop yield, we will be capable of developing more resistant genotypes, enhancing their resilience to hostile situations with minimum energy costs, which can be directed to the development of parts of commercial interest, such as seeds. Below, we describe how aluminium (Al^{3+}) toxicity, P deficiency and drought affect nodulation and N_2 fixation in legumes and the perspectives to create plants that can sustain high levels of N_2 fixation even during adverse situations.

9.2 Legume nitrogen fixation under drought stress

Legume nitrogen fixation is affected by a range of abiotic stresses, for example, high salt, drought, extreme temperature, flooding and aluminium toxicity. Among these, the effect of drought on N fixation has been studied most extensively. Soil water limitation inhibits nodule initiation, nodule growth and development, as well as nodule function (Serraj *et al.*, 1999a; Vadez *et al.*, 2000; Streeter, 2003; Pimratch *et al.*, 2008). Legumes that export ureides (allantoin and allantoic acid, such as soybean, cowpea and pigeon pea) in the nodule xylem are generally more sensitive to drought stress than those that export amides (principally glutamine and asparagine, such as alfalfa, faba or broad bean, chickpea and pea (Sinclair & Serraj, 1995)). A small portion of legumes export both ureides and amides in the nodule xylem, for example common bean (Sprent, 1980).

Drought stress exerts complex and systemic effects on plants, and in nodulated legumes, the complexity is even more pronounced. SNF occurs in the

bacteroid which resides inside the root cells. Based on the reaction catalysed by nitrogenase, under drought stress, several factors may alter the nitrogenase activity: (1) decreased ATP stock, which could be caused by reduced carbon supply from the plant, or by lower O_2 concentration, which restricts respiration efficiency; (2) feedback inhibition by NH_3, H_2, adenosine diphosphate (ADP) or P*i*; (3) altered pH gradient across the bacteroid membrane; (4) nitrogenase activity regulation by substrate affinity or availability, or gene expression. Among these factors, previous research and reviews (Arrese-Igor *et al.*, 1999; Serraj *et al.*, 1999a; Serraj, 2003) focused mainly on three aspects – C source limitation, O_2 restriction and N feedback inhibition – which is also the focus of the current review. In addition, several relating factors affecting nitrogen fixation under drought stress are briefly discussed.

9.2.1 Carbon restriction

It has long been known that plant photosynthesis rate decreases under drought stress, and earlier studies demonstrated an association between N_2 fixation rate decrease and leaf photosynthesis decline (Huang *et al.*, 1975). However, N_2 fixation is extremely sensitive to drought stress (Apariciotejo & Sanchezdiaz, 1982; Weisz *et al.*, 1985; Fellows *et al.*, 1987; Sheoran *et al.*, 1988; Djekoun & Planchon, 1991; Streeter, 2003; Galvez *et al.*, 2005; Ladrera *et al.*, 2007), with nitrogen fixation decline often preceding the decline in leaf photosynthesis (Durand *et al.*, 1987; Djekoun & Planchon, 1991; Verdoy *et al.*, 2004), which means that the decrease in nodule C demand precedes the decline in plant C supply. The amount of sucrose and total non-structural carbohydrates in the nodule was repetitively shown to sharply increase under drought stress in many legumes (e.g. Gonzalez *et al.*, 1998; Ramos *et al.*, 1999; Galvez *et al.*, 2005; Naya *et al.*, 2007), especially in soybean (Fellows *et al.*, 1987; Gonzalez *et al.*, 1995, 1998; Serraj *et al.*, 1998; Streeter, 2003; Naya *et al.*, 2007).

Being a strong sink, nodules actively fixing N_2 require a constant supply of photosynthates by the host plant through the phloem. Sucrose is translocated from source organs to the nodules and subsequently metabolized in the infected cells to produce malate, thereby supporting the bacteroids. In nodules, sucrose synthase (SS), rather than alkaline invertase, is the primary enzyme that breaks down sucrose (reviewed in Arrese-Igor *et al.*, 1999; Horst *et al.*, 2007). The importance of SS in soybean nodules under drought stress was first demonstrated by Gonzalez *et al.* (1995); after a mild and gradual water stress by withholding soil water (nodule water potential, ψ_w, decreased from -0.6 to -1.2 MPa in 4 days), SS activity decreased significantly accompanying the decline in the N_2 fixation rates, while the activity of other enzymes unchanged, for example, alkaline invertase, phosphoenolpyruvate carboxylase (PEPC) and glutamine synthetase (GS). The rapid decline of nodule SS activity during drought stress was later confirmed in soybean (Gordon *et al.*, 1997; Ladrera *et al.*, 2007) and other amide-exporting legumes (Galvez *et al.*, 2005; Marino *et al.*, 2007; Naya *et al.*, 2007; Marino *et al.*, 2008).

Supported by unambiguous evidence, sucrose does not appear to be a limiting factor in drought-stressed nodules. However, how nodule malate biosynthesis, which is a product of the TCA cycle, affected by sucrose accumulation as well as relating enzyme activity changes remains an open question. Several studies reported a significant decrease in nodule malate concentration under drought stress associated with a decline of SS activity in pea (Galvez *et al.*, 2005; Marino *et al.*, 2007) and soybean (Ladrera *et al.*, 2007). However, opposite observation was made by Naya *et al.* (2007) in alfalfa under severe stress. The reason for this discrepancy is unclear and does not seem to be associated with nodule amide or ureide-exporting characters.

Finally, is sucrose accumulation in nodule cells under drought stress a consequence of water limitation or an adaptive response to drought stress? Sucrose has been shown to accumulate in a variety of plants under stress to serve as osmoticum to maintain normal cellular functions. In drought-stressed nodules, it could serve a similar role. Supporting evidence for this hypothesis includes the concomitant decrease in starch concentration in drought-stressed nodules (Gonzalez *et al.*, 1995; Gordon *et al.*, 1997; Ramos *et al.*, 1999) even though more studies are needed in this area.

9.2.2 Nodule permeability to O_2

To maintain a relatively stable and extremely low pO_2 in the infected zone, nodule permeability is self-regulatory to certain extent under a variety of environmental conditions. For example, when the rhizosphere pO_2 was artificially raised, nodule permeability decreases to keep the internal pO_2 relatively stable to avoid damages to the N_2 fixation machinery (Serraj *et al.*, 1995).

The alteration of nodule O_2 permeability under drought stress was described early on. Sprent (1972) observed structural changes in water-stressed, detached soybean nodules. The most pronounced changes were seen in vacuolated nodule cells with folding and dehydration of the cell wall, particularly around air spaces, disruption of the plasma membrane and organelles, and plasmolysis was also observed. In broad bean (Guerin *et al.*, 1990) (Fig. 9.2) as well as common bean (Verdoy *et al.*, 2004), nodules from water-deprived plants showed clear cell wall folding and apparently diminished air spaces in the cortical cells. In addition, in drought-stressed common bean, Ramos *et al.* (2003) observed damage of bacteroid and peribacteroid membranes, senescence of bacteroids, and occlusion of the intercellular air spaces with cell-released material and bacteria.

The O_2 diffusion coefficient is about 10 000 times faster in air than in water (Hunt *et al.*, 1988); the collapse and reduction of the interconnected air spaces in the nodule therefore greatly increase the air diffusion resistance. Increased dehydration of the air spaces and the cell walls was explained by several hypotheses, for example the accumulation of glycoproteins in the intercellular spaces of the inner cortex (James *et al.*, 1991; Delorenzo *et al.*, 1993;

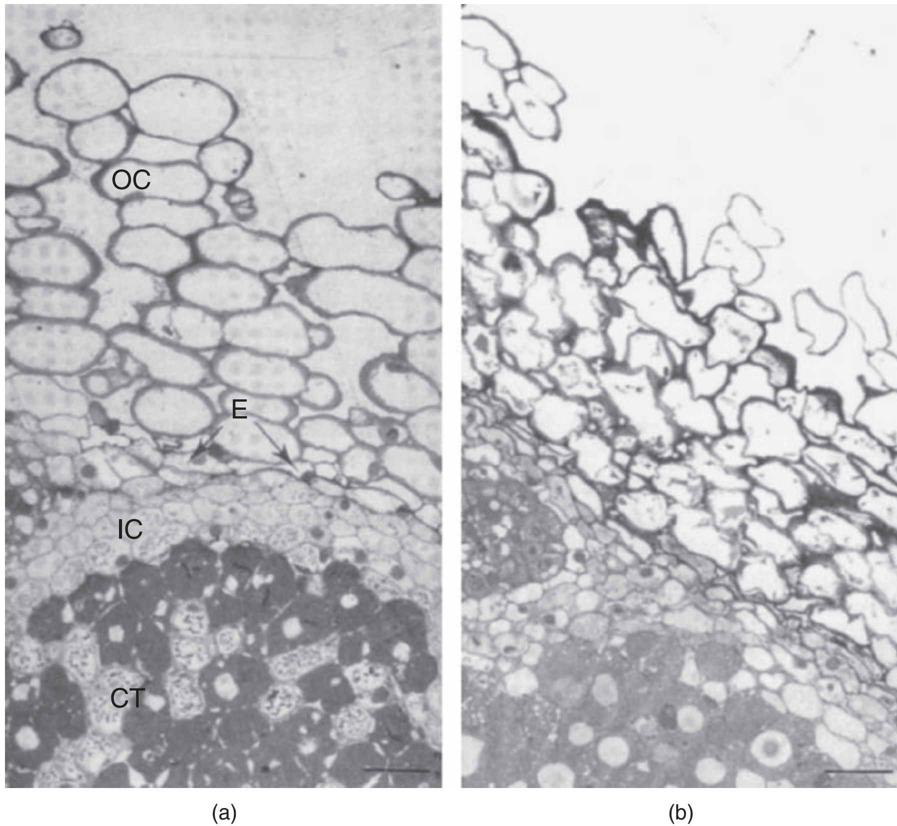


Figure 9.2 Light micrographs of sections of broad bean nodules. Sections were from the functional part of nodules from well-watered (a) and water-deprived (b) plants. OC, outer cortex; E, endodermis; IC, inner cortex; CT, central tissue. Bars indicate 10 μm. Reproduced from Guerin *et al.* (1990), with permission from the American Society of Plant Biologists.

Vandenbosch *et al.*, 1994; Verdoy *et al.*, 2004). The inner cortex cells may act as osmocontractile cells mimicking pulvinic or guard cells (Serraj *et al.*, 1995; Drevon *et al.*, 1998), and potassium flux might be involved in the swelling and contraction of the cortex cells (Wei & Layzell, 2006).

O_2 limitation could reduce the bacteroid respiration rate, thus also the SNF. Alternatively, low pO_2 may protect nodule cells from oxidative damage when the respiration rate is low due to metabolic restrictions. Several studies demonstrated that SNF rate decline under drought stress was associated with a reduction in the nodule O_2 permeability (e.g. Pankhurst & Sprent, 1975; Weisz *et al.*, 1985; Durand *et al.*, 1987). However, there was also evidence showing that the decrease in nodule respiration rates and SNF rates preceded the decline in nodule pO_2 under drought stress (Diaz Del Castillo *et al.*, 1994;

Diaz Del Castillo & Layzell, 1995) or in PEG-treated, hydroponically cultured soybean plants (Purcell & Sinclair, 1995). Nonetheless, elevated rhizosphere pO_2 was shown to restore nodule nitrogenase activity under mild, but not severe, water stress (Serraj & Sinclair, 1996a, 1996b).

In summary, it is evident that nodule O_2 permeability decreases under drought stress. However, how and to what extent this decline affects SNF is not clear. In addition, the role of reduced nodule permeability is probably only significant under mild or moderate drought stress.

9.2.3 Nitrogen feedback regulation

Nitrogen metabolism plays an important role in regulating SNF under drought stress. As mentioned earlier, nodule ureide-exporting legumes are generally more drought-sensitive than amide exporters (Serraj *et al.*, 1995). When legume plants are drought-stressed, SNF rate decreases rapidly; however, rather than observing a shortage of N compounds in the plant, ureides were found to accumulate in both nodules and shoots in drought-stressed plants (Serraj & Sinclair, 1996b; Serraj *et al.*, 1999b; Vadez *et al.*, 2000; Charlson *et al.*, 2009). Several reasons could account for this phenomenon: decline in shoot N demand, lower xylem translocation rate due to a decreased transpiration rate, or a relative decline of metabolic enzyme activity.

Earlier works supported N feedback inhibition from the accumulation of shoot ureides. Vadez & Sinclair (2001) examined nine soybean genotypes and revealed a positive relationship between shoot ureide concentrations and drought sensitivity. In addition, the drought-sensitive cultivars 'Biloxi' and 'Williams' showed higher ureide concentrations in shoots under drought stress than the drought-tolerant 'Jackson' (Serraj & Sinclair, 1996b; Purcell *et al.*, 1998; Charlson *et al.*, 2009). However, using the same 'Biloxi' and 'Jackson' genotypes, Ladrera *et al.* (2007) did not detect significant leaf ureide accumulation for either cultivar under drought stress, while nodule ureide concentrations were more closely correlated with a decline in the SNF rate. With a split-root system in pea, Marino *et al.* (2007) demonstrated that under drought stress SNF activity is mainly controlled locally rather than through a systemic signal generated in the shoots.

Compared with ureide-exporting legumes, studies on N feedback regulation of SNF in amide-exporting legumes are scarce despite their fundamental metabolic differences. In ureide-exporting legumes (Fig. 9.3), uric acid is synthesized in the infected nodule cells via the purine synthesis pathway using NH_3 as N source, which is the direct product of SNF. Next, uric acid is transported to uninfected cells, where it is used to synthesize allantoin and allantoic acid. In nodule amide-exporting legumes, glutamine and asparagine are believed to be synthesized in the infected cells. However, it is unknown how ureides and amides are then transported to the xylem. Are they being released to the apoplast by the synthesizing cells and then move apoplastically to the xylem, or, do they diffuse symplastically through plasmodesmata

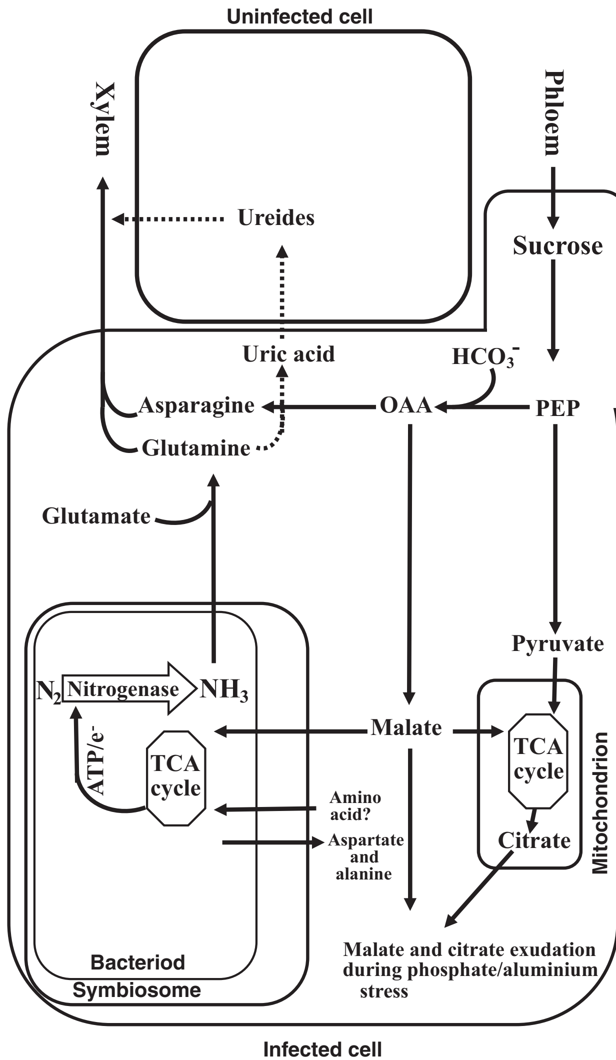


Figure 9.3 Generalized scheme of symbiotic nitrogen fixation in the nodules of ureide and amino acid (amide) exporting legumes. Solid arrows indicate organic acid and amino acid metabolism and dashed arrows indicate ureide metabolism (provided by Dr Yun Kang).

and subsequently being released near the xylem? This information is critical because it may resolve the exact location where ureides or amides accumulate in the nodule under drought stress. It is well known that ureides have low solubility in water (~35 mM at 20°C; Sprent, 1980), and that the whole ureide biosynthesis pathway occurs in different cell types, with transport of intermediates. Therefore, although nodule ureides consistently accumulate to high concentrations under drought stress, N feedback inhibition is probably more

directly related to the amount of soluble amides and amino acids in the nodule. Indeed, for hydroponically grown soybean plants, 10 mM asparagine caused a greater and faster reduction in SNF than 10 mM allantoic acid (Serraj *et al.*, 1999b). A number of reports showed elevated levels of asparagine, glutamine and/or total amino acids in drought-stressed soybean nodules in addition to ureides (Gordon *et al.*, 1997; Streeter, 2003; King & Purcell, 2005).

For amide-exporting legumes, it is intuitive to expect a stronger N feedback regulation on SNF since amide synthesis and N_2 fixation occur in the same cells, and that glutamine and asparagine are both highly soluble. However, to date, very few measurements were made on amide levels in drought-stressed nodules of amide-exporting legumes, although a slight increase in total amino acids was observed for pea nodules (Gonzalez *et al.*, 1998). Galvez *et al.* (2005) characterized C/N interactions in pea nodules under mild drought stress (leaf $\psi_w > -1.2$ MPa) and found a significant decline in activity of GS and aspartate aminotransferase (AAT), which could be a sign of feedback inhibition of glutamine and asparagine accumulation; however, the concentration of glutamine or asparagine was not measured. In alfalfa, another amide exporter, glutamate synthase (GOGAT) activity, decreased significantly under severe stress (leaf $\psi_w = -2.1$ MPa) but not under moderate stress (leaf $\psi_w = -1.3$ MPa) (Naya *et al.*, 2007). Again, amide concentrations in the nodules were not determined. Interestingly, similar decline in enzyme activity was also observed for GS and GOGAT in ureide-exporting legumes (Ramos *et al.*, 1999; Figueiredo *et al.*, 2007). Thus, it would be interesting to monitor the nodule amide concentration changes in drought-stressed amide-exporting legumes to test whether the N feedback regulation is universal for all legumes.

9.2.4 Additional factors affecting SNF under drought stress

9.2.4.1 Leghaemoglobin

Leghaemoglobin (Lb) is a unique plant haemoglobin and indispensable for SNF to occur in nodules. Lb concentrations can reach 2–3 mM in the cytoplasm of infected cells (Bergersen, 1982), but Lb changes in studies to characterize nodule responses to drought stress have been highly inconsistent. Nandwal *et al.* (1991) measured total Lb in pigeon pea nodules and found a rapid decrease (from 2.8 to 0.7 mg/g dry weight in vegetative plants) associating the soil ψ_w decline (from −0.37 to −1.34 MPa) and nitrogenase activity decline. Similar results were obtained by Guerin *et al.* (1990) in broad bean. In accordance with these reports, Gordon *et al.* (1997) revealed in soybean nodules a clear drop in Lb transcripts when the midday leaf ψ_w dropped to −1.4 MPa. However, opposite observations were also reported. In soybean, Lb amount (mg Lb per mg protein) was kept constant when the nodule ψ_w dropped to −1.2 MPa (Gonzalez *et al.*, 1995). In pea, the quantity of Lb shown in Western immunoblots was the same between the control- and drought-stressed plants, with midday leaf ψ_w of −1.1 MPa (Gonzalez *et al.*, 1998). In alfalfa (Naya

et al., 2007), Lb transcripts did not change even when the leaf ψ_w dropped to −2.1 MPa. Finally, Lb transcription was shown to be up-regulated by 1.8-fold in soybean in a suppression subtractive hybridization library approach when the nodule ψ_w dropped to −3.0 MPa (Clement *et al.*, 2008). From these highly inconsistent results, it is hard to draw a conclusion of how Lb is regulated during drought stress. These results may reflect the possible species or cultivar variation in Lb response to drought stress, or the importance of experimental conditions affecting Lb metabolism.

9.2.4.2 Other gases besides O_2

As discussed earlier, nodule permeability to O_2 decreases under drought stress. In fact, when this occurs, nodules are probably affected by lower diffusion of other gases as well. In this case, could N_2 be limiting for SNF? Could CO_2 be limiting for PEPC activity? Could H_2 accumulate to levels that induce feedback inhibition of nitrogenase activity? So far, very little attention has been raised on these questions except for a model built by Hunt *et al.* (1988) on gas exchange and diffusion in nodules. In this model, an aqueous barrier was assumed to exist in the nodule cortex and the thickness of the barrier can be regulated to maintain an O_2 concentration of 10 nM in infected cells. The rhizosphere pO_2 varied between 10 and 100 kPa, and the corresponding CO_2, N_2 and H_2 concentrations were predicted. Although these hypothetical conditions were not identical to what happens during drought stress, the concept of a variable aqueous layer is valid. In addition, under high pO_2, an increased thickness of the aqueous layer as well as decreased nodule permeability mimics what occurs in the nodule under drought stress. In this model, N_2 was predicted to be not limiting probably because the N_2 consumption rate is far below that of O_2 in infected cells. The predicted N_2 concentration was over 100 μM, which is well above the maximum K_m (N_2) for nitrogenase isolated from *Azotobacter*, *Clostridium* and *Klebsiella* (Li & Burris, 1983). The effective concentration of HCO_3^- was predicted to be about 5.5 mM in infected cells, which is approximately 14 times the K_m (HCO_3^-) of PEPC and is not limiting. The argument was that CO_2 has both higher diffusion rates and water solubility than O_2, N_2 or H_2; therefore, the retardation of CO_2 diffusion when the air space diminishes is probably not as significant. Lastly, the model predicted that the concentration of H_2 in infected cells was probably inhibitory to N_2 fixation under normal growth conditions, and this effect increases when the nodule permeability decreases.

9.2.4.3 Bacteroid

Among the many investigations on legume responses to drought stress, the other indispensible component in the nodule symbiosis, the rhizobia, has often been neglected. Guerin *et al.* (1990) showed that bacteroids isolated from drought-stressed nodules had significantly lower N_2 fixation ability compared with those from unstressed nodules. This result implies that drought stress caused functional impairment of the rhizobia, which was not directly

relevant to the metabolism or physical changes in the root cells. Additional work raised the importance of proline metabolism in the bacteroids under drought stress (Zhu *et al*., 1992; Kohl *et al*., 1994; Gonzalez *et al*., 1998; Curtis *et al*., 2004). Bacteroids were demonstrated to be able to metabolize proline as one C source besides malate in abiotically stressed nodules, and this feature could be important in the maintenance of the SNF machinery and the rhizobia survival under unfavourable conditions. Finally, the communication between rhizobia and the host plant may be critical under water-deficient conditions. Athar and Johnson (1996) did cross inoculations among three alfalfa accessions and seven rhizobia strains, demonstrating clearly that the optimal combination between the alfalfa accession and the rhizobia strain could markedly increase both the nodulation rate and the SNF ability under drought stress. Extending this information to other legume crops could be very helpful in dry-land agricultural practices.

9.2.4.4 Abscisic acid

Abscisic acid (ABA) is considered the most important plant hormone regarding the plant response to drought. Despite this, the role of ABA in nodule function has not been extensively explored. Cho and Harper (1993) showed that application of ABA to soybean decreased the nodule number and weight. Gonzalez *et al.* (2001) reported that ABA drastically reduced SNF and in particularly decreased Lb content. Ding *et al.* (2008) showed that ABA could inhibit nodulation, rhizobial infection and gene expression of several nodule-associated genes. Linking the physiological and biochemical processes discussed with ABA signalling will be one of the central challenges in this area, and will help to understand how the legumes adjust their nodule metabolism in response to abiotic stress. Genetic approaches, such as the identification of ABA-insensitive mutants from legumes, would provide useful tools for answering questions in this area.

9.2.5 Final remarks on SNF drought stress

The effects of drought stress on SNF have been studied extensively since 1970s. Earlier works focused primarily on N_2 fixation rate decline, nodule number reduction, as well as decreases in nodule O_2 permeability under drought stress. Later, more attention was raised on the C and N metabolism regulation of SNF as well as oxidative stress under drought. So far, a complex interaction of physiological, biochemical and genetic regulations has been observed in drought-stressed legumes, though how to integrate these changes in one big picture is a challenge that molecular and system biologists are expected to take in order to answer, such as the exact sequence of the events mentioned above and which factors are decisive to confer drought tolerance.

In addition, the research on signalling transduction pathway in the nodule under drought stress is nearly blank, and many questions remain to be addressed in this area. For example, if the sucrose synthase gene is among

the earliest genes whose expression is altered in the nodules under drought stress, what is the signal trigging the expression of sucrose synthase gene? What contributes to the different drought stress sensitivity between ureide- and amide-exporting legumes? In-depth molecular studies are needed to answer these questions.

Finally, from the evolutionary point of view, it seems beneficial for a legume plant to turn off SNF under stress, since N_2 fixation is energetically expensive and not vital. However, could a *super* legume be generated to produce both determinate and indeterminate nodules and switch between these two developmental types under different environmental conditions? Although this may sound implausible, nature has proved its feasibility with *Sesbania rostrata* (Fernandez-Lopez *et al.*, 1998).

9.3 Soil acidity

The ice-free land area of the earth consists of up to 30% acid soils (Haynes & Mokolobate, 2001; Akinrade *et al.*, 2006), which stretch from the northern cold temperate region to the southern tropical region (von Uexkull & Mutert, 1995; Akinrade *et al.*, 2006). A feature of these acid soils is that nutrient toxicity and deficiency often occur together, such as the toxic levels of aluminium (Al) and manganese (Mn), while phosphorus (P), nitrogen (N) and magnesium (Mg) are deficient (Kochian *et al.*, 2004; Liao *et al.*, 2006). In these soils, Al and P are the most important limitations to plant production, due to their ubiquitous distribution (Kochian *et al.*, 2004; Liao *et al.*, 2006), and both Al toxicity and P deficiency have been reported to have major effects on plant growth in acid soils (Clark, 1977; Delhaize & Ryan, 1995; Zheng *et al.*, 1998a, 1998b; Ligaba *et al.*, 2004).

In an agricultural context, approximately 40% of the world's arable land is considered to be acidic, where P deficiency is commonly reported together with Al toxicity (Clark, 1977; Ligaba *et al.*, 2004) and both have been considered as being inseparable factors that limit crop productivity in these soils (Kochian, 1995). In spite of Al forming 7% of the earth crust, it only becomes toxic to plants in acidic soils when the pH is less than 5 and the Al^{3+} form becomes soluble (Delhaize & Ryan, 1995; Kochian, 1995; Zheng *et al.*, 1998a, 1998b; Ligaba *et al.*, 2004). P is quite abundant in many soils, but because it forms insoluble complexes with cations and is bound to organic compounds by microbial action (Vance, 2001), it remains largely unavailable for plant uptake (Bieleski, 1973; Schactman *et al.*, 1998). In acid-weathered soils this problem may be exacerbated, especially in tropical and subtropical regions of the world (Vance, 2001; von Uexkull & Mutert, 1995).

For legumes, soil acidity is a major constraint to growth and productivity in many of agricultural systems around the world (Graham, 1992; Clarke *et al.*, 1993; Bordeleau & Prevost, 1994; Correa & Barneix, 1997), and the impacts

of both Al toxicity and P deficiency on SNF should be considered as major limitations on the legume contribution to the global N cycle.

9.3.1 Aluminium toxicity

The toxic Al form, Al^{3+}, only becomes soluble and detrimental to plants in soils with pH less than 5 (Delhaize & Ryan, 1995; Kochian, 1995; Zheng *et al.*, 1998a, 1998b; Ligaba *et al.*, 2004). Although legumes have been the subject of many studies on Al^{3+} toxicity, very few have concentrated on the effects of Al^{3+} toxicity on SNF and related factors that can affect nodule function. Tolerant plants are thought to counteract Al^{3+} toxicity via chelation of Al^{3+} ions by organic acids, either internally within the cell or externally via organic acid exudation (Delhaize *et al.*, 1993; Delhaize & Ryan, 1995; Ryan *et al.*, 1995; Jones, 1998; Ma *et al.*, 2001; Ryan & Delhaize, 2001).

For the legumes, altered organic acid metabolism and exudation have been found in a variety of species. In soybean, increased citrate exudation is proposed as a major mechanism for Al^{3+} toxicity tolerance (Silva *et al.*, 2001; Yang *et al.*, 2001), which is also in agreement with a role for increased citrate metabolism during Al^{3+} toxicity for common bean (Miyasaka *et al.*, 1991). In soybean, there is an interaction between Al^{3+} toxicity and P availability in that Al^{3+}-induced citrate exudation can be increased by short-term P deficiency, but not long-term exposure (Nian *et al.*, 2003). Further studies on the P and Al^{3+} toxicity interaction in soybean have shown that, although the P-efficient genotypes are more tolerant to Al^{3+} toxicity due to increased organic acid exudation, the combination of P deficiency and Al^{3+} toxicity in these genotypes can lead to a reduction in organic acid exudation. In alfalfa, the overexpression of nodule-enhanced organic acid-synthesizing enzymes, malate dehydrogenase (MDH) and PEPC, has resulted in higher accumulation and exudation of organic acids, thereby conferring Al^{3+} tolerance to root tips (Tesfaye *et al.*, 2001). Further studies with transgenic alfalfa, where bacterial citrate synthase was expressed in roots, found that Al^{3+} tolerance of root tips was greatly enhanced (Barone *et al.*, 2008).

In spite of the elucidation of altered organic acid metabolism during legume exposure Al^{3+}, there are no studies to show how this diversion of organic acids towards Al^{3+} toxicity may affect nitrogen fixation in legumes. The role of Al^{3+} toxicity in affecting SNF is summarized in Table 9.1 and is evident in a variety of nodule responses, including nitrogen derived from atmosphere (NDFA) percentage, direct effects on the bacterial symbiont and nodule development.

9.3.2 Aluminium and symbiotic nitrogen fixation

Al^{3+} toxicity studies have revealed varied responses in field and glasshouse studies. These studies have measured the SNF or the capacity of SNF in various means, such as using ^{15}N-isotope feeding, acetylene reduction assay or SNF export products in the xylem sap.

Table 9.1 The effect of Al^{3+} on nodule development and nodule function

Al^{3+} effect	Legume species	References
	Nitrogen fixation	
Reduced	*Trifolium subterraneum* L.	Unkovich *et al*. (1996)
Reduced	*Trifolium subterraneum* L.	Sanford *et al*. (1994)
No change	*Trifolium subterraneum* L	Peoples *et al.* (1995)
No change	*Phaseolus vulgaris*	Franco and Munns (1982)
Reduced	*Glycine max*	Silva and Sodek (1997)
Reduced	*Trifolium repens* L.	Jarvis and Hatch (1985)
	Nodulation/rhizobial growth	
Reduced	*Trifolium subterraneum* L.	Unkovich *et al*. (1996)
Reduced	*Trifolium subterraneum* L.	Sanford *et al*. (1994)
Reduced	*Vigna unguiculata*	Hohenberg and Munns (1984)
Reduced	*Stylosanthes* spp.	de Carvalho *et al.* (1981)
Reduced	*Stylosanthes* spp.	de Carvalho *et al.* (1982)
No change	*Phaseolus vulgaris*	Franco and Munns (1982)
Reduced	*Glycine max*	Munns *et al.* (1981)
Reduced	*Glycine max*	Sartain and Kamprath (1975)
No change	*Phaseolus vulgaris*	Franco and Munns (1982)
Reduced	*Trifolium repens*	Jarvis and Hatch (1985)
Reduced	*Trifolium* spp.	Wood *et al.* (1984)
Reduced	*Glycine max*	Silva and Sodek (1997)

Information is sourced from selected publications, where the effects of Al^{3+} are listed as an increase, decrease, no change for nitrogen fixation and nodulation/rhizobial growth.

In the field, soil acidity and high Al concentrations can reduce the dependence of legumes on SNF and increase their reliance on soil N utilization (Unkovich *et al.*, 1996). In acidic soils with relatively high Al concentration, such as the south-western Australian pastures, it was found that liming can significantly increase the SNF performance of subterranean clover, probably by reducing the availability of Al^{3+} at a higher pH (Sanford *et al.*, 1994). In certain cases where liming of acidic soils had no effect on SNF, *Trifolium subterraneum* may have been resistant to Al, due to the already high dependence (90%) on SNF (Peoples *et al.*, 1995). Under field conditions, the forms of Al that are available to the plant and responses of the plant to these Al species should be taken into account regarding the effect of Al on SNF (Sprent, 1999). This is based on findings that certain legumes can grow in the high Al soils of the Brazilian savanna are able to nodulate and fix N_2 in the field (Goedert, 1983; Sprent *et al.*, 1996).

In studies conducted under controlled conditions, high Al concentrations have varied effects on SNF for a range of *Rhizobium* strains and host legumes. In one study with common beans colonized by *Rhizobium phaseoli*, the capacity for SNF did not change with Al exposure, measured as nitrogenase activity via the acetylene reduction assay (Franco & Munns, 1982). In this study, it seems clear that the symbiont strain, *R. phaseoli*, was more resistant than the host, because it was less affected than root and shoot growth (Franco &

Munns, 1982). The sensitivity of the symbiont strain may differ with the species of the host, and SNF may be more susceptible to Al^{3+} toxicity than nodule growth. Such was the case in *Trifolium repens*, where Al^{3+} toxicity greatly reduced SNF at Al concentrations where nodule initiation was also inhibited, but also at low levels of Al^{3+} concentrations that did not affect nodulation (Jarvis & Hatch, 1985).

In soybean, the exposure of nodules to Al caused more than 90% reduction in SNF, measured as xylem sap ureide levels, and this was ascribed to the decline in nodule mass under these conditions (Silva & Sodek, 1997). In striking contrast to this decline in ureide concentration, there was an increase in amino acid levels in the xylem sap, but it was attributed to the greatly reduced exudation rate often observed with Al treatments (Silva & Sodek, 1997). Furthermore, during Al exposure, there was also an alteration in the amino acid composition of the xylem sap, especially for the two most abundant amino acids, asparagine and glutamine (Pate *et al.*, 1980; Silva & Sodek, 1997). During Al^{3+} toxicity in this soybean study, the increase in asparagine levels (from 30 to 49 mole percent), whilst glutamine levels declined (from 23 to 8 mole percent), caused an increase in asparagine/glutamine ratio, which may be indicative of a reduced dependence on SNF for N provision to the host (Pate *et al.*, 1980; Silva & Sodek, 1997).

Whether field or glasshouse studies are considered, the mechanism by which Al^{3+} toxicity can reduce SNF remains largely unknown. However, it is likely that the inhibition of SNF by Al^{3+} may occur via similar physiological mechanisms as in roots, where Al^{3+} toxicity can result from complex interactions with cell wall, plasma membrane and cytosolic targets (Kochian *et al.*, 2005). Although other mechanisms may be involved, the major role of citrate, malate and oxalate production during Al^{3+} toxicity in legumes (Silva *et al.*, 2001; Tesfaye *et al.*, 2001; Yang *et al.*, 2001; Nian *et al.*, 2003; Barone *et al.*, 2008) may divert these organic acids away from nodule metabolism (Fig. 9.3). It is known that an alteration in organic acid metabolism, during other conditions of abiotic stress (P deficiency) in nodules, can greatly impact nodular SNF and amino acid metabolism (Le Roux *et al.*, 2006; Le Roux *et al.*, 2008).

Based on the findings of organic acid metabolism and SNF (Le Roux *et al.*, 2006; Le Roux *et al.*, 2008), it seems that the host plant may be an important target for tolerance to high Al soils. Although it has been found that tolerant host species and *Rhizobium* strains are required for reducing the impact of Al and acid soil constraints on nodulation and SNF (Vargas & Graham, 1988), other studies have suggested that SNF tolerance to high Al soils can be achieved by manipulating the host legume, rather than the *Rhizobium* (Taylor *et al.*, 1990; Bordeleau & Prevost, 1994).

9.3.3 Aluminium and soil-borne rhizobia

The reduction of SNF by Al exposure, may also occur at the level of the rhizobia symbionts, either as free-living bacteria or as bacteroids in the nodule

symbiosis. It is known that N-fertilized legumes are less susceptible than legumes that are dependent on SNF, because of the sensitivity of *Rhizobium* to acidic conditions (Sartain & Kamprath, 1975; Andrew, 1976; Hohenberg & Munns, 1984).

However, several Al-resistant strains of rhizobia in different culture environments and symbiotic hosts have been identified (Zahran, 1999). A rhizobial strain with Al resistance has been isolated in the ureide-exporting legume, *Phaseolus vulgaris* (Vargas & Graham, 1988), as well as the amino acid exporting legume, *Lotus* spp. (Wood *et al.*, 1988). The mechanism of Al interaction with rhizobia appears to be at the genetic level, because it has been found that Al can suppress the expression of nodulation genes in *Rhizobium leguminosarum* bv. *trifolii* (Richardson *et al.*, 1988). Furthermore, the DNA has been identified as a possible site for Al interaction with *Rhizobium* spp., but that DNA synthesis was not affected in tolerant species (Johnson & Wood, 1990).

9.3.4 Aluminium and nodule development

The effect of Al^{3+} toxicity on SNF may also occur via the inhibition of nodule growth and development. Al^{3+} can inhibit nodulation directly or indirectly by stunting root growth (Bordeleau & Prevost, 1994; Silva & Sodek, 1997). The direct effects of Al^{3+} on nodulation may occur by preventing the roots from nodulating (Bell *et al.*, 1989) or by delaying the onset and development of nodulation (Alva *et al.*, 1990; Bordeleau & Prevost, 1994).

It has been suggested that, during the long-term exposure of legumes to Al, the nodulation may merely be delayed and that normal growth and development can be achieved with time (Franco & Munns, 1982). This was found for *Stylosanthes* spp. (de Carvalho *et al.*, 1981; Franco & Munns, 1982) and common beans where nodules were abundant during the flowering stage of plants (Franco & Day, 1980; Franco & Munns, 1982). This delay in nodulation may be more important in legumes with determinate rather than indeterminate nodules (Franco & Munns, 1982). This is evident in the case of common beans, where their short life cycles and decline in SNF during the early pod filling stage (Franco & Munns, 1982) can cause a reduction in the amount of N derived from SNF, because SNF may not be maximized in the early stages of the life cycle of legumes with determinate nodules (Franco & Munns, 1982).

9.3.5 Final remarks on aluminium and SNF

In summary, the high levels of Al can reduce SNF in various ways, from the inhibition of rhizobial growth in the soil to the retardation of nodulation to the possible alteration in organic acid metabolism (Table 9.1). Although it is well known that Al^{3+} toxicity can affect association between the legume host and bacterial symbiont at any of the infections stages (Jarvis &

Hatch, 1985), the effect of Al^{3+} on N derived from SNF has been the subject of fewer investigations and should be a target for future work.

9.4 Phosphate deficiency

Phosphorus is abundant in many soils, but is largely unavailable for plant uptake (Bieleski, 1973; Schactman *et al.*, 1998), with further limitations on availability imposed by acid-weathered soils (von Uexkull & Mutert, 1995; Vance, 2001), and the increasing use of P fertilizer placing a strain on inexpensive rock P reserves (Vance, 2001). According to some estimates, these rock P reserves could be depleted in 60–80 years (Council for Agricultural Science and Technology, 1988; Runge-Metzger, 1995; Vance, 2001).

For legumes, P limitation is one of the most important environmental constraints (Jakobsen, 1985; Israel, 1987; Høgh-Jensen *et al.*, 2002; Le Roux *et al.*, 2009), and the role of P in limiting SNF involves a complex suite of mechanisms that may interact at various levels of nodule structural and functional organization. In this regard, the separate effects of P on nodule dry mass, adenylate/P*i* levels and carbon metabolism, should be considered, but moreover their interacting and entwined networks cannot be excluded.

9.4.1 P deficiency and nodulation

P deficiency can reduce nodule growth due to the high nodular requirement for P, but the effect of P deficiency may be directly as P required for nodule growth and metabolism, or indirectly as an effect of carbon supply to the nodules.

It has been reported that nodules often respond relatively slower to P deficiency (Israel, 1993), as evidenced by the delayed decline of P*i* in nodules only 25 days after P starvation (Le Roux *et al.*, 2008), compared to a previous study where 14 days of P withdrawal had no apparent effect on P*i* concentrations of nodules (Le Roux *et al.*, 2006). During the prolonged P starvation period (25 days), the decrease of nodule weight and P concentrations concurs with previous reports on nodule growth and P levels during P deficiency (Drevon & Hartwig, 1997; Oliveira *et al.*, 2004). Using *Lupinus angustifolius*, Le Roux *et al.* (2008) found that the carbon construction costs of these nodules were unaffected and, since construction costs represent the amount of simple carbohydrate required to build new tissue (Peng *et al.*, 1993), this implies that new nodule growth was limited by P supply and not by C allocation. P deficiency may not always limit nodule growth, and during short-term P deficiency, the growth of nodules can be maintained even at the expense of root growth, as found in soybean (Le Roux *et al.*, 2009), *Lupinus angustifolius* (Le Roux *et al.*, 2006, 2009) and *T. repens* (Høgh-Jensen *et al.*, 2002). A possible effect of P limitation can be mediated via carbon supply to nodules, and

during P deficiency, the reductions in shoot growth and photosynthesis can thereby limit nodule growth (Jakobsen, 1985; Almeida *et al.*, 2000).

The effect of P deficiency on nodule growth will also depend on the periods of P starvation. During prolonged P limitation, the mechanism of nodule growth inhibition appears to operate by directly inhibiting nodulation, or by indirectly reducing C supply.

9.4.2 P deficiency and adenylate/P*i* levels

Legumes relying on N_2 fixation require more P than when N is acquired from soil mineral N (Sa & Israel, 1991; Ribet & Drevon, 1995; Al-Niemi *et al.*, 1997, 1998; Tang *et al.*, 2001). The influence of P on SNF in leguminous plants has received considerable attention (Israel, 1987), but its role in maintaining nodule metabolism remains unclear. From previous studies on nodule P*i* levels during P deficiency, it seems that the period of P deprivation is critical to the nodule responses (Le Roux *et al.*, 2006, 2008).

It has been observed that soybeans grown with fertilizer nitrogen have a lower P requirement than when nitrogen is obtained from SNF (Leidi & Rodríguez-Navarro, 2000). Additionally, it was demonstrated that nodules can take up P from the host, but do not readily release its P reserves to the host root (Al-Niemi *et al.*, 1998), suggesting that nodules form strong sinks for P. This important role of P in nodules is supported by the threefold higher P concentrations compared to other tissues (Vadez *et al.*, 1997). Therefore, under conditions of P limitation, the optimum symbiotic interaction between the host plant and rhizobia would depend on efficient allocation and the use of available P (Al-Niemi *et al.*, 1997, 1998).

During short-term exposure to P stress, the root component of legumes may experience P deficiency more severely than nodules (Le Roux *et al.*, 2006). These findings concur with previous P-deficiency studies of cellular P*i* decreases and an ATP decline relative to ADP in roots (Freeden *et al.*, 1989; Theodorou *et al.*, 1991). In contrast to roots, the nodule can maintain constant P*i* levels and ADP–ATP ratios during short-term exposure to P stress, amid the fluctuations of root P*i* and adenylates.

The reason why nodules may be able to maintain their levels of P*i* and adenylates, during short-term P deficiency, is because they may function optimally at the low P*i* concentrations, as supported by previous findings (Al-Niemi *et al.*, 1997, 1998; Colebatch *et al.*, 2004; Le Roux *et al.*, 2006). It therefore appears that nodules may have a strategy to regulate P influx, allowing nodules to minimize effects of P deficiency when supply is low (Jakobsen, 1985; Tang *et al.*, 2001).

The bacteroid fraction of the nodule may also be able to control its response to P stress in the nodule. From physiological studies (Sa & Israel, 1991; Al-Niemi *et al.*, 1997, 1998), it is clear that bacteroids in the nodule consistently operate at low P concentrations and can fulfil their P requirements by scavenging from the host cells (Al-Niemi *et al.*, 1997, 1998).

9.4.3 P deficiency and carbon metabolism

It is well known that legumes relying on SNF have a greater P requirement than when N is acquired from soil mineral N (Sa & Israel, 1991; Ribet & Drevon, 1995; Al-Niemi *et al.*, 1997, 1998; Tang *et al.*, 2001). The high-sensitivity SNF to environmental conditions may be attributed to the C costs (Mengel, 1994; Le Roux *et al.*, 2006, 2008; Le Roux *et al.*, 2009), and for functional nodules, this higher requirement of P may be linked to its role in nodule carbon and energy metabolism, with the plant cell fraction possibly being more energy limited under low P supply (Sa & Israel, 1991; Le Roux *et al.*, 2009). It should be noted that the responses of nodule carbon metabolism are very species-dependent, which may reflect the differences in legumes from tropical and temperate origins, determinate and indeterminate nodules, and amino acid and ureide exporters (Table 9.2). Furthermore, the different responses are also dependent on the duration of P deficiency, as this was variable between 2 and 4 weeks in the different studies.

Table 9.2 The effects of phosphate (P) deficiency in nodules on biological nitrogen fixation, as mediated by nodule weight, adenylate/P*i* levels and carbon metabolism

P deficiency	Legume species	References
	Nodule weight	
Reduced	*Lupinus angustifolius*	Le Roux *et al*. (2006)
Reduced	*Lupinus angustifolius*	Le Roux *et al*. (2008)
No change	*Glycine max*	Le Roux *et al*. (2009)
Reduced	*Lupinus angustifolius*	Le Roux *et al*. (2009)
Reduced	*Trifolium repens*	Almeida *et al*. (2000)
Reduced	*Trifolium repens*	Høgh-Jensen *et al*. (2002)
No change	*Lupinus albus*	Schulze *et al*. (2006)
	Adenylate/P*i* levels	
Reduced	*Lupinus angustifolius*	Le Roux *et al*. (2006)
Reduced	*Lupinus angustifolius*	Le Roux *et al*. (2008)
Reduced	*Glycine max*	Sa and Israel (1991)
No change	*Glycine max*	Le Roux *et al*. (2009)
Reduced	*Phaseolus vulgaris*	Oliveira *et al*. (2004)
No change	*Lupinus angustifolius*	Le Roux *et al*. (2009)
	Carbon metabolism	
No change (OA; AA)	*Lupinus angustifolius*	Le Roux *et al*. (2006)
Increased (OA)	*Lupinus angustifolius*	Le Roux *et al*. (2008)
Reduced (AA)	*Lupinus angustifolius*	Le Roux *et al*. (2008)
No change (CC)	*Lupinus angustifolius*	Le Roux *et al*. (2008)
Reduced (GR)	*Lupinus angustifolius*	Le Roux *et al*. (2008)
Reduced (CC; GR)	*Glycine max*	Le Roux *et al*. (2009)
No change (AA)	*Phaseolus vulgaris*	Oliveira *et al*. (2004)
Increase (OA)	*Lupinus albus*	Schulze *et al*. (2006)

Information is sourced from selected publications, where the effects of P deficiency are listed as increased, reduced and no change for nodule weight, adenylate/P*i* levels and carbon metabolism. OA, organic acids; AA, amino acids; CC, carbon construction cost; GR, carbon growth respiratory cost.

Although studies on carbon metabolism in P-deficient legume nodules are scant, the responses of non-legumes to limiting P supply in the growth medium are well known. These responses range from an increase in root carbohydrate content (Rychter & Randall, 1994) to lowered rates of total respiration rate and root ATP concentrations (Rychter *et al.*, 1992; Wanke *et al.*, 1998). At the metabolic level for non-legumes, an important response to P deprivation is organic acid metabolism via alternative routes (Duff *et al.*, 1989; Theodorou & Plaxton, 1993) to facilitate mitochondrial respiration by P*i*-deficient plant cells (Plaxton, 1996; Sieger *et al.*, 2005). In these cases, a major point of divergence in glycolysis is at phosphoenolpyruvate (PEP) metabolism which involves the engagement of non-adenylate, requiring steps via the sequential action of MDH and PEPC activities. This serves to circumvent the conventional adenylate requiring pyruvate kinase route.

For nodules under P stress, a potential drawback at this PEP branch point would be the constant competition for C skeletons between the organic acid pool and the amino acid pool (Fig. 9.3) in the nodules (Oliveira *et al.*, 2004; Le Roux *et al.*, 2006). This is because the major C substrates for bacteroid respiration and N_2 fixation are dicarboxylic acids, primarily in the form of malate (Rosendahl *et al.*, 1990). High malate levels in nodules are indicative of the importance of this particular metabolite (Streeter, 1987; Rosendahl *et al.*, 1990), and metabolomics and transcriptomics analyses indicate that elevated levels of malate in root nodules are mainly the result of very low oxygen and phosphorus concentrations (Colebatch *et al.*, 2004).

In a study with short-term P deficiency in *L. angustifolius*, Le Roux *et al.* (2006) found that high nodular malate concentration was associated with enhanced MDH and PEPC activities, even though the nodules were not experiencing P stress. Using ^{14}C-labelling, Le Roux *et al.* (2006) further found that short-term P deficiency did not alter the pyruvate that was derived from malate via the malic enzyme. These findings are in agreement with a study in soybean nodules (McCloud *et al.*, 2001), and suggest that PEP metabolism is favoured via the PEPC and MDH route to ensure high malate levels in nodules. Under these conditions, it is likely that malate is a major C source for bacteroid respiration inside the nodules (Rosendahl *et al.*, 1990). It has been proposed that the low oxygen concentration in nodules would favour malate, rather than pyruvate as the main end product of glycolysis (Vance & Heichel, 1991).

However, when the period of P deficiency in *L. angustifolius* was extended until the nodules experienced P stress, Le Roux *et al.*, (2008) found in a follow-up study that the engagement of non-adenylate, requiring steps via the sequential action of PEPC and MDH, caused an even greater increase in malate concentrations in the nodules. This served to circumvent the conventional adenylate requiring pyruvate kinase route and thereby ensured the continuation of respiration. However, the resulting high malate concentrations in the nodules caused a decline in SNF and a shift in C allocation away from amino acid synthesis and towards organic acids.

Apart from affecting organic acid metabolism, P stress in nodules can also directly alter N metabolism. There is evidence that P deficiency may affect nodule SNF via N feedback mechanisms. During P deficiency, the accumulation of asparagine in xylem sap, roots and nodules occurred with the reduction in SNF for *T. repens* in two separate studies (Almeida *et al.*, 2000; Høgh-Jensen *et al.*, 2002). In these studies, it was suggested that the P limitation on SNF may operate via a reduction in host growth, which would consequently require less N. Evidence that P deficiency in nodules may directly affect N metabolism has been found in the initial assimilation step of N_2 to NH_4^+ being more sensitive to P deficiency, but sensitivity was gradually reduced with increasing P nutrition of the nodule (Israel & Rufty, 1988). Oliveira *et al.* (2004) reported that in P-stressed *P. vulgaris* nodules, the NH_4^+ assimilation in nodules was shifted from ureide to amine (asparagine) production in this ureide-exporting legume. This was supported enzyme activities, where GS was unaffected, AAT increased and uricase (urate oxidase) decreased, during P deficiency (Oliveira *et al.*, 2004). These findings of a shift in the export products under P stress concur with Le Roux *et al.* (2009) for another ureide exporter, soybean. However, using nodule metabolite levels, Le Roux *et al.* (2009) found that more ureides were exported compared to amino acids under P stress. Although these two studies do not concur regarding the changes in SNF export products, these findings highlight the difficulty of comparisons between studies where different methodologies and P starvation periods were employed. Nonetheless, they still serve to indicate that shifts in N metabolism can occur in P-deficient nodules.

The studies regarding the alterations in nodule organic acid and amino acid metabolism under P deficiency offer merely a limited view into the intricate metabolic regulation of nodule metabolism. Much work is needed to extend these findings, not only for fundamental understanding of the metabolic flexibility, but also for breeding purposes where legumes can be selected for efficient N metabolism under perturbations in P supply.

9.4.4 P deficiency and oxygen diffusion

The effect of low P on O_2 diffusion into the nodule is a striking and intriguing feature of the nodule adaptation to P deficiency. This is because effective SNF is primarily regulated by the diffusion and availability of oxygen into the infected cell zones of nodules (Layzell *et al.*, 1990; Hunt & Layzell, 1993; Schulze *et al.*, 2006). P starvation can affect nodule O_2 permeability for both amide- and ureide-exporting nodule types (Ribet & Drevon, 1995; Drevon & Hartwig, 1997; Schulze & Drevon, 2005; Le Roux *et al.*, 2009), but does not appear to cause a down-regulation of nitrogenase activity (Schulze, 2004).

The mechanism of P deficiency in affecting the oxygen diffusion barrier appears to be an increase of O_2 diffusion into the nodules under P stress, as seen for example in soybean (Ribet & Drevon, 1995), common bean (Vadez *et al.*, 1996) and alfalfa (Schulze & Drevon, 2005). Although Drevon *et al.* (1998)

proposed that the O_2 diffusion may be facilitated by osmoregulatory changes in the size of nodule cortical cells, other legume species have been shown to use a different strategy of regulating O_2 diffusion. In *Lupinus albus*, it was reported that O_2 diffusion in nodules is controlled by the occlusion of the free spaces between the cortical cells (De Lorenzo *et al.*, 1993; Iannetta *et al.*, 1993; Schulze *et al.*, 2006). In addition, P-deficient plants form smaller nodules, which may also facilitate O_2 diffusion by the increased nodule surface area/nodule volume ratio, as evidenced by the increased respiration rates and the SNF remaining unchanged (Ribet & Drevon, 1995). The physiological role of increased O_2 uptake under P deficiency remains obscure, but it has been suggested that it may be related to maintaining a sufficient adenylate energy charge for the high SNF rates (Schulze & Drevon, 2005).

In summary, the impact of P deficiency on nodule SNF is an important feature of acidic soils. The nodular adaptations to P deficiency cover a wide range of developmental, physiological and biochemical alterations. These adaptations form a suite of interacting responses that are primed for not only different periods of P starvation but also different species and bacterial symbiont selections.

9.4.5 Final remarks on P deficiency and SNF

The global distribution of acid soils in natural and managed ecosystems is a major problem to legume SNF. Of all the nutrient stresses that may occur on these soils, Al toxicity and P deficiency are the most important limitations to legume SNF and therefore the ability of legumes to contribute to the global N cycle. Although both Al toxicity and P deficiency frequently co-occur on acid soils, not all plants that appear to be resistant to both. For legumes, a goal for tolerance should be the breeding or genetic modification of both host and bacterial symbionts for tolerance to Al toxicity and P deficiency.

9.5 Legume biology is taking off

Much of what is known today on plant molecular biology comes from basic studies carried out in the model species *Arabidopsis*, including on stress responses. Indeed, this species has been ideal to fast forward many aspects of plant biology, since it is small, proliferous, has a fast life cycle, and holds a simple, small genome. However, *Arabidopsis* does not cover all aspects of plant biology, and other models are required (Benedito, 2007). For example, 80% of land species of plants can establish a mycorrhizal symbiotic relationship, whereas *Arabidopsis* cannot. Other examples are specific secondary metabolism routes, organ architecture, flowering biology, fruit development, as well as SNF.

The last common ancestor between *Arabidopsis* (Rosid II clade) and legumes (Rosid I) is reckoned to have existed approximately 90–100 million years ago

(Wikström *et al.*, 2001; Yan *et al.*, 2003). Nowadays, legumes encompass an important angiosperm family, being the third largest family in the plant kingdom (with 19 300 species, only after Orchidaceae and Asteraceae), and the second most important crop family in the world, only after grasses (Poaceae). In basic studies, this plant family is now gaining momentum with the near completion of three genome-sequencing projects (soybean, and the models *L. japonicus* and *M. truncatula*), making it possible to understand how legumes evolved and function at the molecular and genetic levels, enabling the manipulation of traits of economical importance, such as those leading to higher yields or improved nutritional values.

These genome sequences will provide ample opportunities to produce tools for genetic breeding programmes and will expand our basic understanding on SNF to a great extent. Mutant populations, such as a comprehensive *Tnt*1 insertional population (Tadege *et al.*, 2005, 2008) as well as a fast neutron bombardment deletion population (Rujin Chen, personal communication), both being largely expanded at the Noble Foundation (http://bioinfo4.noble.org/mutant/), will be of great importance to unravel gene functions in many aspects of legume and plant biology. Proteomics (Mathesius *et al.*, 2001; Catalano *et al.*, 2004; Lei *et al.*, 2005; Oehrle *et al.*, 2008; Tian *et al.*, 2009) and metabolomics approaches (Desbrosses *et al.*, 2005; Farag *et al.*, 2008) have been applied to several legume species, but the picture in nodules under several developmental, physiological or genetic conditions is still turbid (Prayitno *et al.*, 2006; Larrainzar *et al.*, 2007). Although these techniques are not restricted to previous knowledge of the genome, enabling its application to any species, they are greatly facilitated by the creation of comprehensive metabolic libraries (Broeckling *et al.*, 2006), peptide mass fingerprint database, biochemical pathway databases (Urbanczyk-Wochniak & Sumner, 2007), and the prior knowledge of the full conceptual proteome derived from the genome sequence. Comprehensive transcriptome resources are available to elucidate gene expression modulation under diverse environmental conditions and developmental situations: the Medicago Gene Atlas (Benedito *et al.*, 2008; http://bioinfo.noble.org/gene-atlas) (Fig. 9.4) that will help elucidate gene interactions and networks to better explore the potential that legumes can add to plant biology as well as crop production.

9.6 Beyond genomics: prospects for legume genetic breeding

Although molecular resources in legumes have been spent mostly on two models – *M. truncatula* and *L. japonicus* – and soybean, many other species in this family are very relevant ecologically and economically, providing feed, food and drinks (e.g. beans, carob, lentils, lupins, peas, red bush and tamarind), forage (alfalfa, clover, stylo), timber and shade (acacias, mesquite,

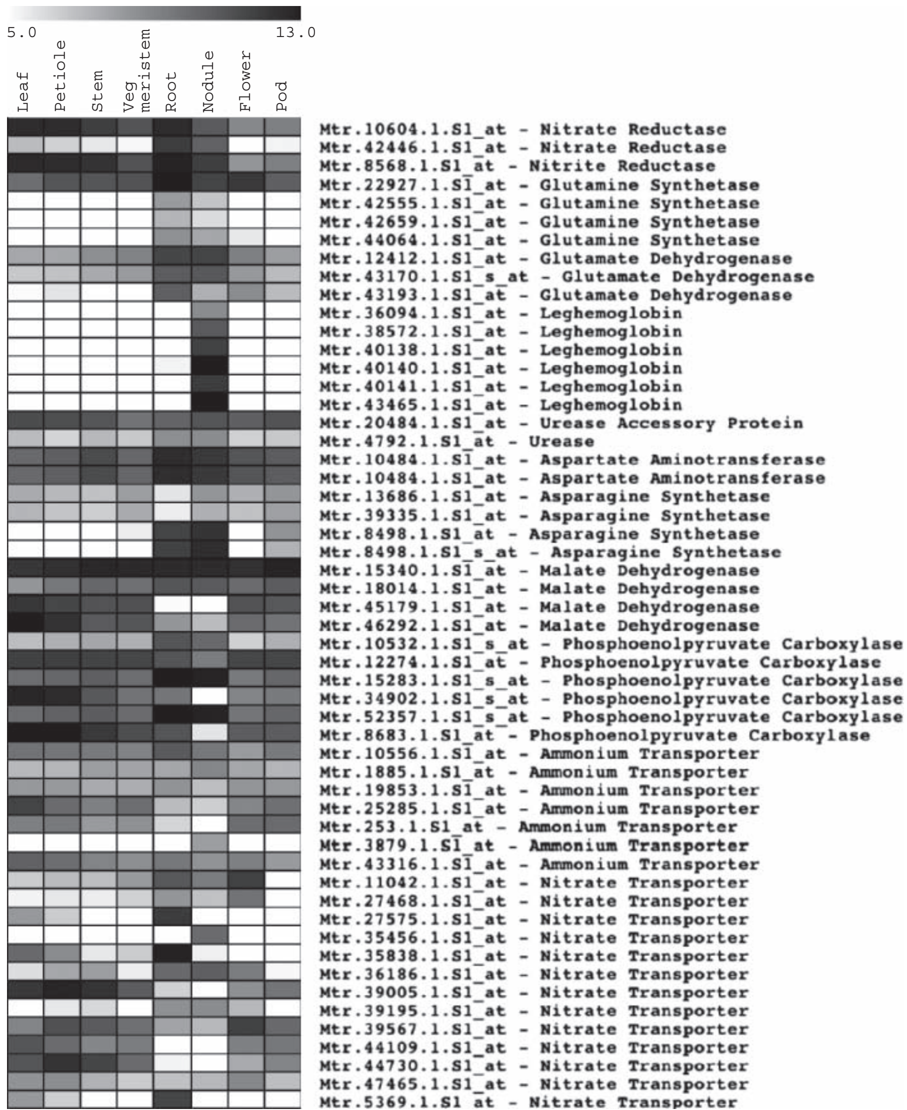


Figure 9.4 Gene expression levels in several organs of *M. truncatula* under optimal growth conditions. Several genes coding for enzymes involved in nitrogen metabolism and symbiotic nitrogen fixation are depicted. Note that several alleles may redundantly code for isozymes, granting plasticity and possibly, resilience, to the biological system. Studies of nodules under stresses will certainly increase our understanding of symbiotic nitrogen fixation at the molecular level. The dataset is acquired from the Medicago Gene Atlas (Benedito *et al.*, 2008; http://bioinfo.noble.org/gene-atlas) and log_2-transformed for display. Graphic visualization was produced with TMEV programme.

locust), gum (guar), as well as populating ecosystems and playing important roles in the food chain, and the cycling of N and other nutrients (Doyle & Luckow, 2003). So far, no attempt was made to explore the molecular genetics beyond the Faboideae (Papilionoideae) subfamily, which encompasses all pulse legumes.

Our understanding of N fixation in legumes has improved much in recent years: symbiotic signalling cascades are being unravelled (including common factors to other symbioses, such as mycorrhization); the genetic players of nodule development programmes are being functionally characterized (e.g. transcription factors and miRNAs); biochemical pathways involved in chemical cross-talk between symbionts and metabolism are becoming more comprehensive; and symbiotic membrane transporters are being intensively studied at the moment.

And indeed, by using the genome sequences of available model legume species, gene-derived molecular markers are being produced based on comparative genetic mapping to explore syntenic regions in diverse legume species, including for the so-called orphan crops (Choi *et al.*, 2004, 2006; Zhu *et al.*, 2005; Varshney *et al.*, 2009; Young & Udvardi, 2009). Extensive synteny has already been revealed among legume species (Cannon *et al.*, 2006), which will allow sequenced legume genomes to be used as scaffold for subsequent assembling the genome of more thwarting species (Gepts *et al.*, 2005), since more cost/time-efficient DNA sequencing techniques will soon allow to be directly applied to orphan species.

The challenge now is to integrate functional genomics to SNF under stress conditions to understand adaptation, robustness and resilience not only of legumes but also of N_2 symbiosis. This is essential to provide useful information to be translated to crop breeding programmes. Also, understanding how agronomic traits are developed at the molecular level will enhance our understanding on how to manipulate them in a more efficient way, so that breeders could have available molecular tools to advance their programmes in a fast-forward way (Young & Udvardi, 2009), not necessarily only by using genetic engineering, but also via marker-assisted selection of alleles conferring advantageous phenotypes.

Yet, comparative legume genomics tools are being made available and will largely expand once model genome sequences are consolidated and orphan species start to integrate the dataset. Examples of such platforms with a myriad of analysis tools are the Legume Information System (http://www.comparative-legumes.org) and Legoo – Integrative Legume Resources (http://www.legoo.org) as well as the resource platforms and databases aforementioned.

Molecular tools currently under development (*-omics* platforms and databases, gene association, mutant populations, physical maps and diverse markers) will help functional genetics approaches to foster cultivar development. Detecting how gene expression changes under stress and identifying the principal components driving these changes through genetic web

construction is enhancing our comprehension of stress biology and possible factors to tackle to increase stress tolerance. Understanding how SNF is performed under stress and how to overcome it in order to keep high nitrogenase activities with minimal extra energy cost will potentially increase crop yields via biotechnological tools in breeding programmes. That is where the next leap in the legume genomics revolution lies.

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Chapter 10

METABOLOMICS APPROACHES TO ADVANCE UNDERSTANDING OF NITROGEN ASSIMILATION AND CARBON–NITROGEN INTERACTIONS

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Abstract: In the past 10 years, metabolomics has become an integrative and indispensable part of the post-genomics approach to tackle questions on plant metabolism and its regulation in plants. Nonetheless, the dynamic range of the metabolic intermediates and their qualitative (chemical) diversity hampers the comprehensive analysis of the plant metabolome using one single platform. However, prompted by the increasing interest in routine analysis of the metabolic component, recent significant advances in experimental techniques, analytical technologies and tools for the elaboration of output data have been accomplished. Hence, our capability to identify an increasing number of metabolites and to integrate results from different approaches has greatly improved. Specific to the study of plant nitrogen metabolism, the introduction of metabolomics, alongside with transcriptomics, and metabolic flux analysis emphasized the complexity of the mechanisms regulating its assimilation and metabolism and the indissoluble

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interaction with carbon metabolism. In this chapter, we introduce the state of the art in metabolomics analytical tools and technologies as well as their employment to improve our understanding of nitrogen–carbon interaction in plants.

Keywords: alkaloids; cytokinins; gas chromatography–mass spectroscopy; glutamate synthase; glutamine synthetase; jasmonic acid; metabolomics platforms; nicotine; nuclear magnetic resonance; 2-oxoglutarate; 2-oxoglutarate dehydrogenase; phenylpropanoids; phosphoglucomutase; quadropole and time-of-flight techniques

10.1 Introduction

The N status of a plant determines the composition of its primary and secondary metabolites including amino acids, proteins, carbohydrates, polyamines, phenylpropanoids, terpenoids and hormones. In turn, these changes both directly and indirectly affect crop yield and quality traits such as the protein and oil content of seeds. In the past two decades, the evolution of increasingly more sensitive and accurate high-throughput technologies for the analysis of the levels of protein transcripts and metabolites has resulted in an unprecedented volume of data with which we can address important biological questions such as definition of the regulatory processes which govern N assimilation and C–N interactions. In parallel with the development of these analytical platforms, major advances in plant genetics have been achieved. Completed genome-sequencing projects of crops such as rice, sorghum and grape, alongside nascent sequencing programmes of maize, *Medicago*, potato and tomato, as well as the recent establishment of population of recombinant inbred (RIL) and introgression lines (IL) suggest that there is a great and realistic potential that we can define the cardinal players with respect to nitrate use efficiency.

The combination of genetic variability and high-throughput analyses provides scientists with an incredible array of tools with which to study biological processes, their significance and regulation. In the 'post-genomic' era, this is best realized by as comprehensive an analysis of the molecular entities of the cell as possible. Such an approach allows multilevel network analysis and may well uncover important regulatory elements which have, to date, been hidden. The growing number of metabolomics-related research articles, and the widening spectrum of science fields within which this approach is being integrated, reflects its considerable role within systems biology. In turn, these factors prompt and justify the ongoing investment in refining present analytic platforms and developing further protocols, displaying more sensitive and thus comprehensive capabilities for precise and reliable metabolite identification and quantification. In this chapter, we summarize recent advances towards a better comprehension of both the nitrogen assimilatory process and the carbon–nitrogen interactions in plants, paying particular attention to

those for which the use of metabolomics platforms played a part in defining regulatory mechanisms.

10.2 Methods for analysing the plant metabolome

Plant metabolomics is a dynamically evolving branch of science. Routine analysis of the plant metabolome is complicated by the vast number of metabolites present in a given organism that display significant quantitative and qualitative (chemical) diversity (Stitt & Fernie, 2003; Kopka *et al.*, 2004). Whilst there is no single analytical platform capable for acquiring exhaustive information of the metabolic complexity of a biological sample, recent advances in the experimental techniques currently applied as well as the increasing size of libraries for supervised metabolic profiling have greatly improved both aspects of the sensitivity of the analysis and thus have broadened the spectrum of identifiable metabolites. Nuclear magnetic resonance (NMR), mass spectroscopy (MS) and the latest orbitrap-based techniques enable profiling of the levels of a broad number of metabolites from complex biological samples as well as various degrees of structural identification of purified metabolites (Mesnard & Ratcliffe, 2005). However, none of the currently applied methods is free from drawbacks. Although MS offers higher selectivity and sensitivity, it also requires sample preparation steps that may endanger metabolite stability and influence the composition of investigated mixture. Examples of metabolite instability are represented by ascorbate/dehydroascorbate, Glu/pyroGlu and phosphate-containing metabolites. In this regard, a major advantage of NMR is its non-invasive nature of analysis that gives the possibility of *in vivo* analysis of plant tissues, minimizing sample preparation steps. It also allows an observation of the redistribution of the stable isotope label at the atomic level within functioning tissue, which is useful for evaluation of physiological response and the exact calculation of intracellular fluxes. The employment of NMR analysis is however limited to highly abundant metabolites in relatively simply composed mixtures (Dettmer *et al.*, 2007).

Mass spectrometry-based methods are either coupled to separation techniques or used for direct flow injection. The mass spectra complexity can be largely reduced by application of gas or liquid chromatography (GC-MS and LC-MS, respectively) or electrophoresis (CE-MS)-based separation steps, ensuring both high sensitivity and precision of measurements. The combination of gas chromatography with electron-impact ionization MS facilitates quantification of up to 300 metabolites in various plant tissues (Fernie, 2003). However, because it is limited to volatile and thermally stable compounds, chemical derivatization steps are often required prior to sample injection. During the analysis, one must thus be aware of potential artefacts and conversion reactions (Halket *et al.*, 2005) derived from the pre-treatment of the extract ahead of injection. Among three accessible detection methods, quadropole (GC-QUAD) and time-of-flight (GC-TOF) machines are the

most commonly used for plant metabolite profiling, although highly selective ion-trap technology is receiving rising attention for targeted profiling. Also, hybrid instruments, such as quadrupole-TOF-MS, which combine the advantages of both components have been developed and utilized, for instance, for rapid screening of plant extract (Scholz *et al.*, 2004) and for identification of specific mitochondrial proteins involved in carbon metabolism (Warnock *et al.*, 2004). (UP)LC-MS can provide an independent analytical platform as well as one which complements GC-based MS (Fait *et al.*, 2008b). With its high sensitivity and reduced background noise in comparison to classic high-performance liquid chromatography (HPLC), LC-MS is becoming increasingly used in plant science applications. The ability to quantify high-molecular-weight compounds with low polarity is being increasingly employed for the profiling of secondary metabolites in plants (e.g. Sumner *et al.*, 1996; Baumert *et al.*, 2001; Lange *et al.*, 2001; Mellon *et al.*, 2002; Keurentjes, 2009; Matsuda *et al.*, 2009). In the recent years, Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR/MS) has been successfully introduced in several metabolomic strategies (Aharoni *et al.*, 2002; Hirai *et al.*, 2004; Mungur *et al.*, 2005; Dettmer *et al.*, 2007). FT-ICR/MS great advantage lies in obtaining the exact mass measurements with ultra-high resolution and sensitivity in the range of femtomoles, which owes to its extensive resolution and accuracy (Aharoni *et al.*, 2002; Marshall & Hendrickson, 2002; Hirai *et al.*, 2005). That said, only recently (Giavalisco *et al.*, 2008) have the methods been used to acquire data by this strategy been robustly documented, a fact that is a particular concern in any technology that is prone to ion-suppression effects. Both methods are additionally hampered by a lack of authentic chemical standards for large secondary metabolites. By contrast, those for primary metabolites are freely available, as are spectral libraries (see for example Kopka *et al.*, 2005; Luedemann *et al.*, 2008; Tohge & Fernie, 2009), and furthermore, GC-(TOF)-MS maintains a fine compromise between data quality and costs.

10.2.1 Metabolic steady state and flux analysis

GC-MS-based methods can be employed to characterize the qualitative and quantitative changes in metabolite content in a given sample set and provide via so-called steady-state metabolic profiling information on the metabolic phenotype of a biological sample or the metabolic signature of a process (e.g. seed development and biomass accumulation) or a condition (e.g. low temperature acclimation). For example, in a review Hennig has highlighted the role of metabolomics in plant developmental biology to uncover patterns of developmental processes and their regulation (Hennig, 2007). The metabolic signature for high plant growth was analysed in *Arabidopsis* by using an RIL population (Meyer *et al.*, 2007) and led to the identification of metabolic markers for biomass. Metabolomics is being introduced in biogeography studies: the metabolic phenotype within and between populations of

the subalpine perennial plant *Arabidopsis lyrata* ssp. *petraea* was measured for variation across environmental gradients from across its northwest European range. The combination of habitat parameters – genes and metabolic profiles – supported the description of the structure of the distribution of the plant populations (Kunin *et al.*, 2009). In a different study, the effect of the plant–pathogen interaction was measured in the profiles of primary and secondary metabolites in poppy plants (Zulak *et al.*, 2008). The results showed extensive reprogramming of primary metabolism in association with the induction of alkaloid biosynthesis. These and many other recent research papers and reviews emphasize the important role of metabolic profiling platforms in modern plant science research. Nonetheless, the complexity of the biological networks imposes integrative and synergetic analysis in combination with other high-throughput analytical platforms and a robust genetic basis (Keurentjes, 2009). Due to technical limitations, the metabolic phenotype, whilst informative concerning the major metabolic processes occurring in a cell, will cover only a minor fraction of the metabolite complement in a cell (Fernie, 2003; Messerli *et al.*, 2007). Furthermore, it lacks the dynamic component that can be, at least partially, provided in metabolic flux experiments.

Experimentally based approaches to estimate metabolic flux through cells and tissues generally rely either on steady-state isotopic labelling or on kinetic modelling. Whilst the latter demands extensive information on the pathway involved including reaction constants, and enzymatic kinetics, the first relies on the assumption that the system is at steady state and that the label is redistributed at a rate proportional to the *in vivo* metabolic steady-state flux. Another important factor in labelling experiments is the magnitude of the isotopic pulse: the internal pool of the substrate used must be considered to avoid major perturbations (Stephanopoulos *et al.*, 1998; Morgan & Rhodes, 2002). In stable isotope labelling experiments, the isotopes used are non-radioactive (^{2}H, ^{15}N and ^{13}C); hence, NMR spectroscopy or MS can monitor isotopic enrichment in measured metabolites (Ratcliffe & Shachar-Hill, 2001; Roessner-Tunali *et al.*, 2004). Given the assumption that the metabolic system is at steady-state, the labelling experiment requires only a single measurement of abundances of isotopomers of metabolic intermediates or end products. The information on isotope redistribution is usually based on multiple isotopic substrates and coupled with ^{14}C-labelling to provide further information not deducible from ^{13}C-labelling alone, and with steady-state measurements (Dieuaidenoubhani *et al.*, 1995; Fernie *et al.*, 2001) and/or measurements of uptake and excretion from the system (for an extensive review of recent advances in *in-silico* and experimental approaches to metabolic flux analysis, see Schwender *et al.*, 2004; Ratcliffe & Shachar-Hill, 2005; Kruger & Ratcliffe, 2007; Libourel & Shachar-Hill, 2008).

Isotopic labelling has recently been employed on a number of species including *Arabidopsis*, tomato and *Brassica napus*. For example, relative stable isotope redistribution in leaves using a combination of feeding ^{13}C-labelled

substrates to the leaf via the transpiration stream and a recently adapted GC-MS protocol that facilitates the estimation of intracellular fluxes was employed to elucidate C–N interaction in tomato leaves and particularly to understand the relation between Glu metabolism and the tricarboxylic acid (TCA) cycle (Studart-Guimaraes *et al.*, 2007). By evaluating the redistribution of ^{13}C in isolated leaves fed with ^{13}C-Glu, the authors could suggest an up-regulation of Glu catabolism via the γ-amino butyric acid (GABA) shunt, possibly aimed to adjust C–N equilibrium when the TCA cycle is impaired. *In vivo* metabolic fluxes of central C–N metabolism were monitored by steady-state labelling, and measurements of enzymatic activity in cultured developing embryos of *B. napus*. By growing embryos on either organic (Gln and Ala) or inorganic nitrogen source (ammonium nitrate), the authors observed an increase in the anaplerotic flux through phosphoenolpyruvate carboxylase to sustain ammonium assimilation (Junker *et al.*, 2007). The study further demonstrated that embryos can achieve metabolic rearrangements largely without regulatory reprogramming of the enzymatic machinery. In spite of the above and of recent advances in the understanding of the regulatory mechanisms of N assimilation, the actual modulation of metabolic fluxes associated with N assimilation is still under debate partly because of dispersion of data in the literature.

MFA has been successfully employed to elucidate the interplay between primary and secondary metabolism. In a study on the benzoid network in *Petunia*, Orlova *et al.* (2006) employed *in vivo* labelling with $^{2}H_5$-Phe. MFA results suggest an alternative pathway for benzoic acid biosynthesis. An alternative to the labelling of plant samples, the analysis of natural abundance can, in principle, give insights on the origin of atoms as well as on the size of metabolic flux (Badeck *et al.*, 2004; Priault *et al.*, 2009). The latter, however, requires extensive knowledge on the natural isotopic composition of each molecule involved in a reaction and on possible isotopic discrimination in enzymatic reactions (Ledgard *et al.*, 1985) as well as the knowledge of patterns of influx and efflux of the system under study.

10.2.2 Integrative analytical platforms to ameliorate the understanding of plant metabolic pathways

Metabolic profiling has recently been coupled with the development of high-throughput enzymatic essays based on an automated enzymatic cycling system. A robotized system was developed to measure the activities of more than 20 enzymes involved in central carbon and nitrogen metabolism using optimized assays (Gibon *et al.*, 2004). This automated platform for enzymatic reactions measures changes in protein levels as expressed by the change in their maximal catalytic activities (Gibon *et al.*, 2004) and can provide highly valuable information on the turnover dynamics of the cellular metabolism under given conditions. The platform provides a sensitive tool for the quantification of glycolytic intermediates, acetyl-CoA, inorganic pyrophosphate,

nucleotide phosphates and other metabolites that are barely (or not), detectable by the use of mass spectrometry (Gibon *et al.*, 2002). Change in metabolite content as a consequence of a metabolic perturbation, in response to genetic or environmental alteration, will depend on the rates of turnover of the metabolites and of the enzymes governing the metabolic pathways involved in the perturbation. The data obtained can be integrated with changes at the level of transcripts and metabolites and/or with data from isotopic redistribution experiments (Oresic *et al.*, 2004; Gibon *et al.*, 2006; Keurentjes *et al.*, 2008; Usadel *et al.*, 2008). It can be thus superimposed onto metabolic pathway maps such as Kyoto Encyclopedia of Genes and Genomes (KEGG) and eventually assembled to be displayed, on common interfaces (Usadel *et al.*, 2005). Further development of more sensitive analytical methods and, most importantly, of integrative tools aimed at integrating the different data matrices will be crucial to gain full understanding of the complexity of the cellular system and the regulation of N metabolism.

10.3 Uptake and assimilation of nitrate and ammonium

Inorganic N assimilation, its circulation and remobilization are processes of vital importance in plants. Although during the past 50 years, countless experiments have been performed with the aim of elucidating the control of N metabolism, aspects of its control remain unclear. This process starts with the uptake of N from the rhizosphere in the form of nitrate (mostly available) and ammonium (NH_4^+) via specific transporters. Although the latter is also scavenged from the soil via a similar transport system as the one acting for nitrate (Glass *et al.*, 2002), the preferred source of N in C_3 plants is the latter. High amounts of ammonium are generated during photorespiration and phenylpropanoid biosynthesis and its accumulation in the plant cell is considered toxic. Assimilation of nitrate occurs via low-affinity, non-saturable and high-affinity, saturable transport systems operating at mM and μM range of nitrate concentrations, respectively. Plants also possess an inducible nitrate efflux system, although it has much slower turnover rate than the uptake system (Aslam *et al.*, 1996). The sensing of nitrate in the rhizosphere and within the plant thus becomes a highly important task for the plant, and nitrate has been proposed as a biochemical signal to regulate N assimilation and nitrate influx rates (Faure-Rabasse *et al.*, 2002), and growth, including lateral roots and shoot–root ratios (Stitt, 1999; Scheible *et al.*, 2004). Changes in nitrate influx to the root have been illustrated to have long-distance effect on amino acid pool on the flow in the xylem, which in turn modulates the transport of plant hormones between root and shoot (reviewed by Miller *et al.*, 2007).

Once nitrate crosses the plasmalemma, it is either metabolized in the cytoplasm of root cells or transported passively along the gradient of concentration to the shoot via the xylem (Forde, 2002; Orsel *et al.*, 2002). Classic

labelling studies employing ^{15}N-nitrate have shown that in wheat up to 80% of absorbed nitrate is reduced within the leaves (Ashley *et al.*, 1975). Systemic diurnal studies of N assimilation have shown that leaf N metabolism broadly follows a three-step change during the day (Stitt *et al.*, 2002). A first large assimilation of nitrate in the leaves is its reduction to NH_4^+ and glutamine (Gln) with the concomitant accumulation of malate, glycine (Gly) and serine (Ser). Later in the light period, respiration-derived C metabolites accumulate. Finally, during the night, the level of Gln and photorespiratory Gly and Ser decrease dramatically, while the citrate and nitrate pools are replenished. At the cellular level, the reduction of nitrate that follows its acquisition occurs both in cytoplasm and plastids, and it is catalysed by the concerted action of nitrate reductase (NR, NIA) and nitrite reductase (NiR, NII). The resulting ammonium is then primarily incorporated into amino acids through the action of Gln synthetase (GS, GLN) and glutamate synthase (GOGAT, GLU). The carbon skeletons required for this reaction are supplied in the form of 2-oxoglutarate (2-OG). This organic acid acts as the acceptor for ammonium in the GS/GOGAT pathway (Stitt, 1999) and therefore becomes a major interaction point, joining both carbon and nitrogen metabolism. This fact was confirmed recently in potato tubers by evaluating the effect of several inhibitors of 2-OG dehydrogenase on the level of C–N metabolites, metabolic flux and mitochondrial respiration (Araujo *et al.*, 2008). In the light of these results, it is not surprising that, when the metabolic variance present in a tomato introgression line population is measured (Schauer *et al.*, 2006) and data are successively elaborated, 2-OG pattern of change across genotypes resembled that of Ala and other amino acids more than that of other TCA cycle intermediates (Fait *et al.*, 2008a). The tight linkage between C and N metabolism ensures proper acquisition of N and the regulation of its intra- and extracellular levels, eventually modulating plant performance.

10.3.1 N incorporation into primary metabolism

The level of nitrogen compounds throughout the plant reflects the N status of a plant. The concentrations and relative pool content of the latter are indicative of the N partitioning within the plant. Glutamate serves as primary intermediate for the synthesis of most amino acids (Forde & Lea, 2007), and its level is maintained relatively stable across genetic or environmental alteration (Scheible *et al.*, 2000; Matt *et al.*, 2001; Masclaux-Daubresse *et al.*, 2002; Urbanczyk-Wochniak & Fernie, 2005; Urbanczyk-Wochniak *et al.*, 2005; Fritz *et al.*, 2006a). In the understanding of the integration of N in the primary metabolism of the cell, a major limitation is the lack of techniques that enable measurements at the cellular/subcellular level, that is, compartmentalization of N metabolism and the interdependence between N influx, N metabolism and light/photosynthetic activity. A few studies attempted cellular measurements of amino acid regulation of metabolism showing the dependence of amino acid cytosolic metabolism on light (Winter *et al.*, 1994;

Macduff & Bakken, 2003). These and other studies emphasized the tight link between photosynthetic activity, regulation of N status and amino acid pools and available C (Lejay *et al.*, 2003; Noctor *et al.*, 2007) produced via photosynthesis and glycolysis. For example, nitrate reductase (NR) activity and the transcript level respond strongly to external nitrate concentrations and availability of light, sugars and concentration of downstream amino acids (Crawford & Arst, 1993; Lillo, 1994). Moreover, N signals have been shown to induce response of C signals, including sucrose, glucose and trehalose and the previously mentioned 2-OG (Finkelstein & Gibson, 2002; Leon & Sheen, 2003; Palenchar *et al.*, 2004). Finally, Stitt *et al.* (2002) have shown that N assimilation depends on mitochondrial respiration. The latter indeed provides the C skeleton for Glu synthesis, NADH for the NR and ATP for the GS reactions. Recent results on the cytoplasmic male sterile II mutant, which carries the only well-characterized stable mutation in the mitochondrial electron chain, showed a clear effect of lacking mitochondrial complex I on C–N partitioning (Dutilleul *et al.*, 2005), and the mutant also showed altered nitrate sensing phenotype (Pellny *et al.*, 2008). N sensing could be recovered by addition of sugars and giberillic acid, implying a synergistic effect of sugars and hormones (Pellny *et al.*, 2008).

These lines of evidences in the scientific literature are in accordance with more recent findings arising from genomic studies and thus add to new insights into C–N interregulation. A genome-wide transcript analysis in *Arabidopsis* under a set of various conditions of C and/or N combined with tools in global network analysis and qualitative network modelling has emphasized the fact that many genes previously identified as N or C responsive are in fact regulated by some types of C–N interaction (Gutierrez *et al.*, 2007). For example, the study shows that 78% of the nitrate-inducible genes were in fact regulated by N interactions with C, including enzymes and transporters involved in N assimilation functions, such as nitrate transport and nitrate reduction. These results are indicative for a more complex regulatory mechanism of N metabolism than previously suggested.

On a similar line, a systematic approach has been employed to investigate diurnal regulation of C–N metabolism (Gibon *et al.*, 2006). The study included an integrative analysis of 23 enzymes involved in central C and N metabolism, the responses of 137 metabolites, measured using GC-MS and LC-MS and gene expression data. The authors compared wild-type (WT) *Arabidopsis* with *pgm* mutant, lacking plastid phosphoglucomutase activity, essential for photosynthetic starch synthesis (Caspar *et al.*, 1985). In WT plants, starch accumulates in leaves in the light and is degraded to sugars at night. Prolongation of the night leads within a few hours to total exhaustion of starch and a collapse of sugars and related metabolites, even in WT plants (Thimm *et al.*, 2004). In *pgm*, sugars accumulate during the day whilst decreasing to very low levels during the night. These differences raised by the authors question about the adjustment of the metabolic network in recurring accentuated changes in the levels of sugars (Gibon *et al.*, 2006). The results

of this systematic, multileveled study revealed surprising evidences in the complex regulatory interaction of C–N, light signalling and diurnal rhythm. First, transcript levels and enzyme activities responded differently to environmental signals: enzymes' changes in activity due to prolonged darkness were delayed as compared to that of transcripts, and diurnal changes of enzyme activities were not related to changes of the encoding transcript levels. Second, adjustment of metabolism was associated with correlation of transcripts and metabolites; based on the rapid diurnal changes in transcripts, the authors suggest metabolite-driven regulation of gene expression, rather than metabolites level being changed as a consequence of a change in gene expression. Within N metabolism, a co-response to prolonged night and to the loss of plastidial phosphoglucomutase (pPGM) activity was shown at all functional levels, metabolites, enzymatic activities and gene expression: the content of Gln and Glu were lower in the starch-less mutant than in the WT, as were the activities of NR, Gln synthetase and ferredoxin-Glu synthase and transcript levels for the corresponding genes. On the other hand, the levels of minor amino acids increased in a prolonged night and in *pgm*, in line with increased expression of genes associated with amino acid degradation. Although on a less extensive scale, important insights in the regulation of N–C metabolism were shown in a study measuring the effect of N limitation on leaves and seed biomass and metabolism in *Arabidopsis* (Lemaitre *et al.*, 2008). To this aim, the authors employed various methods to determine the level of amino acids, carbohydrates and starch, total N, protein content and N remobilization (^{15}N-labelling experiments) and selected enzymatic essays. Under N-limiting conditions, the level of N remobilization to the seeds increased, leading to a reduced content of residual N in the vegetative part. Via higher N remobilization and recycling pathways, N limitation led to augmented levels of sugars in rosette leaves and less free amino acids. The seed amino acid composition changed in a similar fashion as the rosette leaves in response to N limitation.

The studies here summarized emphasize that a systematic experimental design exhaustively covering N–C conditions and parallel analysis at different cellular levels not only allows to infer quantitative models of gene responses, but aids to detect aspects of N–C interdependent regulation traditionally missed.

10.4 Cross-talk between N and secondary metabolism

The impact of N perturbation is expected to have consequences on secondary metabolites, whose building block can be tracked down to amino acids such as tryptophan (Trp) as is the case for monoterpenoid indole alkaloids or indirectly linked to N pools such as the polyamine-derived tropane alkaloids (Fig. 10.1). The study of the regulation at the interface between primary and secondary metabolism is gaining attention in the recent years, in spite of the – mainly technical – difficulties for comparative studies. Still, the scientific

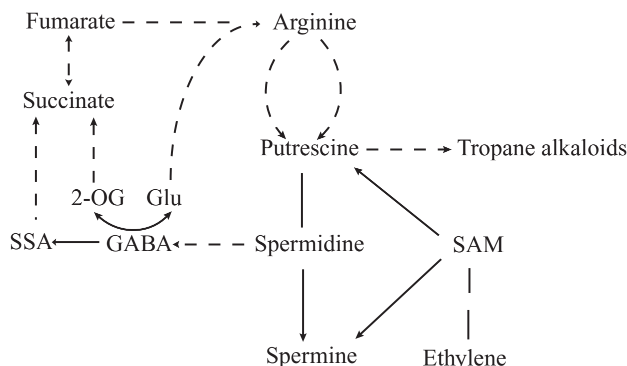

Figure 10.1 Schematic view of a portion of the interface between central C–N metabolism and secondary metabolism (e.g. tropane alkaloids and ethylene).

literature on the global effect of N perturbation on shikimate-derived intermediates remains scant.

Polyamines are ubiquitous, biogenic amines present in most living organisms, and involved in various biological processes. Nonetheless, functional significance of polyamines as well as the molecular basis of their *in vivo* function remains yet to be understood (Kusano *et al.*, 2007). The link with primary N metabolism includes Arg and GABA metabolism (Fig. 10.1; Bagni & Tassoni, 2001; Fait *et al.*, 2008b). Recent successful attempts to modulate their metabolism, by combining genetic engineering and metabolic and transcript profiling, suggest multiple roles for these compounds. Higher polyamines influenced multiple cellular pathways in tomato fruit during ripening (Mattoo *et al.*, 2006). In line with the accumulating evidences for the intermingled relation of N–C metabolism, significant changes were reported in the levels of choline, Glu, Gln, Asn, citrate, malate and fumarate as well as changes in transcripts of key enzymes involved in N–C interactions (Mattoo & Handa, 2008). Interestingly, the authors found commonalities in the metabolic perturbation in mature tomato fruits in response to alteration in polyamine metabolism with the response of roots and leaves to exogenous application of N (Foyer & Noctor, 2002).

Alkaloid biosynthetic pathways, whose members include the anticancer compounds, vinblastine, are produced starting with the conversion of Trp to tryptamine by Trp decarboxylase (for a review on alkaloid metabolism, see Ziegler & Facchini, 2008). NMR-based positional labelling experiments employing stable isotopes of glucose have revealed that triose phosphate/pyruvate and not the mevalonate pathway as generally assumed was the major route for the biosynthesis of the monoterpenoid skeleton (Contin *et al.*, 1998). The first step in tropane alkaloid and nicotine biosynthesis lies in the methylation of the polyamine putrescine to *N*-methylputrescine by putrescine *N*-methyltransferase (Fig. 10.1; Hibi *et al.*, 1994). The recent

establishment of expressed sequence tag (EST) databases in plants including opium poppy, *Encelia californica*, *Camellia japonica* and *Catharanthus roseus* accelerated the studies on the regulation of these important classes of secondary compounds. Given the high content of important alkaloids, opium poppy and *C. roseus* have also been among the first plant species whose metabolome and transcriptome has been profiled in an effort to elucidate the regulation of secondary metabolism. Transcriptome analysis has shown a common induction of the alkaloid biosynthetic pathway by methyl jasmonate (Goossens *et al.*, 2003) and fungal elicitors (Zulak *et al.*, 2007). These changes were accompanied by changes across the primary metabolism (with the peculiar exclusion of N metabolism in opium poppy; Zulak *et al.*, 2007) as represented by the induction of genes implicated in the metabolism of methionine-derived *S*-adenosyl methionine (SAM) and aromatic amino acids (Rischer *et al.*, 2006), which are involved in the structural modification and biosynthesis of alkaloids, respectively. The interaction of N plant status and availability on alkaloids metabolism were further investigated in tobacco (Fritz *et al.*, 2006b). The contribution of nitrate and N metabolism to the regulation of phenylpropanoid and nicotine synthesis was compared in WT and NiR-deficient mutants. By profiling primary as well as secondary metabolites and by integrating the metabolic profiling with microarray analysis and real-time reverse transcription polymerase chain reaction (RT-PCR), the authors monitored the changes of a range of phenylpropanoids and found that these were highly modulated by the genotype versus N source interaction. The metabolic shift was accompanied by parallel changes in relevant enzymes responsible for phenylpropanoid biosynthesis. The differences between opium poppy and tobacco in the interaction of primary N metabolism and secondary compound suggest for different regulatory mechanisms and need further attention. The increasing power of high-throughput profiling supported by multivariate analysis and correlation network visualization is adding insights into secondary metabolism regulation. For example, gene to metabolite network analysis in *C. roseus* has shown synchronic response of metabolites and genes to external signals. Correlation analysis highlighted differences in hormonal regulation of specific branches of terpenoid indole alkaloid biosynthesis. The analysis yielded novel transcript and metabolite tags associated with terpenoid indole alkaloids, as well as their network associations (Rischer *et al.*, 2006). In a plant life cycle, the N is remobilized according to distinct developmental cues. In senescing leaves, N is found largely in the form of ammonium as a result of degradation processes. Ammonium can be then recycled into Glu and subsequently into Gln (Miflin & Habash, 2001). This incorporation is part of the remobilization of N during leaf senescence, which is essential for the development of reproductive tissues. Several metabolic signals have been suggested to be involved in N remobilization, among them are ethylene and cytokinins (Buchanan-Wollaston *et al.*, 2003). The latter is a group of hormones which regulate various biological processes in plants such as cell proliferation and differentiation, senescence and apical

dominance and which were shown to modulate plant senescence, productivity (Gan & Amasino, 1995), as well as N metabolism (Sakakibara *et al.*, 2006). The hypothesis of a tight interaction of N metabolism and cytokinin has been reinforced by the effect displayed by treatments with external N on cytokinin content in several plant species (Singh *et al.*, 1992; Samuelson & Larsson, 1993; Takei *et al.*, 2001). The processes associated with the regulation of leaf sink and source have been studied in soybean (Igarashi *et al.*, 2009). The authors monitored metabolic and gene expression changes during leaf-stage progression and after removal of the reproductive organs. The results from WT and *Arabidopsis* mutants of a cytokinin biosynthetic gene and a cytokinin receptor gene suggest that cytokinins are involved in the cross-talk between the reproductive and the vegetative parts of the plant. Indeed, the hormone has been already suggested as the interchangeable local (Forde, 2002) and long-range (Tamaki & Mercier, 2007) signalling molecule with N molecules (Sakakibara *et al.*, 2006). Furthermore, the study on soybean showed that cytokinin specifically regulates glutamate dehydrogenase (Igarashi *et al.*, 2009), an enzyme involved in N remobilization (Miflin & Habash, 2001) by catalysing a reversible amination–deamination reaction (Melo-Oliveira *et al.*, 1996). These lines of evidence call for more research to disentangle the tight interaction between N and cytokinin role in plant metabolism and development.

10.5 Summary

N metabolism involves N sensing, assimilation and integration into primary metabolism. The regulatory mechanisms involved have been found more complex than previously thought, and C–N interactions have gained increasing attention for their fundamental role in plant response to changes in N availability. A complex regulation of the different processes and their cross-talk at the transcriptional and post-transcriptional levels demands the use of high-throughput technologies to monitor the global changes in transcript, metabolites, proteins and enzymatic activities augmented by the use of systematic experimental design and a robust collection of genetic material (e.g. IL and RIL). Although limited in its application, metabolic flux analysis represents the next level of complexity that research in plant metabolism in general and N metabolism in particular needs to address. The creation of metabolic flux maps via high-throughput experimental flux analysis is still in its infancy. Nonetheless, the increasing convergence of theoretical and experimental approaches in MFA appears promising. Synergism among these different disciplines of investigation is crucial to shed light on the global response of the plant to internal and external alterations in N pool. Whilst the integration of the different omics techniques has ameliorated our understanding in the networks involved in N metabolism, future research will have to address the regulation at the interface of primary and secondary metabolism. Modelling the dynamics of the metabolic network, of which N metabolism is

part of, in response to environmental and endogenous signals will facilitate our understanding of its implication on plant traits of economic value such as crop yield, seed protein content and accumulation of specific secondary metabolites.

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Chapter 11

MORPHOLOGICAL ADAPTATIONS OF *ARABIDOPSIS* ROOTS TO NITROGEN SUPPLY

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Abstract: Higher plants display complex morphological adaptations in their root system to nitrogen availability. In *Arabidopsis*, four such adaptations have been well characterized, which include a stimulatory effect of a localized nitrate supply on lateral root elongation, a systemic inhibitory effect of high nitrate supply on lateral root development, the suppression of lateral root (LR) initiation by high C–N ratios and glutamate-induced regulation of root system architecture. Studies of these adaptations have revealed valuable insights about their underlying mechanisms, in particular those related to N sensing and root development, and also novel regulatory properties of nitrate transporters. The future challenges are to fully understand the mechanisms of the N-related morphological adaptations and to establish whether the adaptations found in *Arabidopsis* and their regulatory mechanisms are conserved in other plants and also the biological significance of the adaptations.

Keywords: *Arabidopsis thaliana*; morphological adaptations; nitrogen (N) availability; roots

11.1 Introduction

Nitrogen (N) availability is a major limiting factor for plant growth and varies considerably in both quantity and spatial/temporal distribution in most soil environments. To maximize their capture of, and also compete effectively for this vital nutrient with other plants and micro-organisms, plants have

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evolved various mechanisms to adapt to fluctuations in N supply, which range from regulation of specific genes to physiological and morphological changes (Wiersum, 1958; Ågren, 1985; Miller & Cramer, 2005; Forde & Walch-Liu, 2009).

Since the classical work of Drew and colleagues in the 1970s (Drew *et al.*, 1973; Drew & Saker, 1975, 1978), morphological adaptations of the root system of higher plants to nutrient availability have attracted considerable research interest. This, together with the use of the model plant *Arabidopsis* as the experimental system in such studies and the availability of new molecular and genetic tools associated with this plant system, has led to significant progresses in the past decade in both identifying new forms of N-related adaptations and understanding their underlying cellular and molecular processes (see reviews by Robinson, 1994; Zhang & Forde, 2000; Forde, 2002; Zhang *et al.*, 2007; Forde & Walch-Liu, 2009). This chapter provides a brief overview of such progress made in *Arabidopsis*.

11.2 N-related morphological adaptations in *Arabidopsis* roots

Before the 1990s, research interest in N-related morphological adaptations focused primarily on the proliferation of lateral roots (LRs) in NO_3^--rich patches in response to a localized NO_3^- supply. A key development in the past decade is the identification and characterization of other forms of N-related morphological adaptations in the root systems of higher plants. Figure 11.1 shows four well-characterized N adaptations in *Arabidopsis* roots, including a localized stimulatory effect of NO_3^- on LR elongation (Zhang & Forde, 1998), a systemic inhibitory effect of high NO_3^- on LR development (Zhang & Forde, 1998; Zhang *et al.*, 1999), a high C–N ratio-induced repression of LR initiation (Malamy & Ryan, 2001; Little *et al.*, 2005) and an L-glutamate-induced regulation of root system architecture (RSA) (Walch-Liu *et al.*, 2006; Walch-Liu & Forde, 2008).

11.2.1 The localized stimulatory effect of NO_3^- on LR elongation

When *Arabidopsis* roots encounter a localized nitrate supply, LR proliferation is significantly stimulated in the nitrate-rich patch (Zhang & Forde, 1998; Zhang *et al.*, 1999) (Fig. 11.2). This response is very similar to that observed by Drew and colleagues in barley seedlings (Drew *et al.*, 1973; Drew & Saker, 1975), except that in *Arabidopsis*, the increased LR proliferation is mainly due to an increase in LR elongation, whilst in barley it is due to a combination of increasing LR initiation and elongation (Fig. 11.3). The response in *Arabidopsis* roots requires a direct exposure of LR tips to NO_3^-, which suggests that the NO_3^- signal is perceived by cells in the LR tips/meristems (Zhang & Forde, 1998, 2000). This response also represents a general adaptive strategy

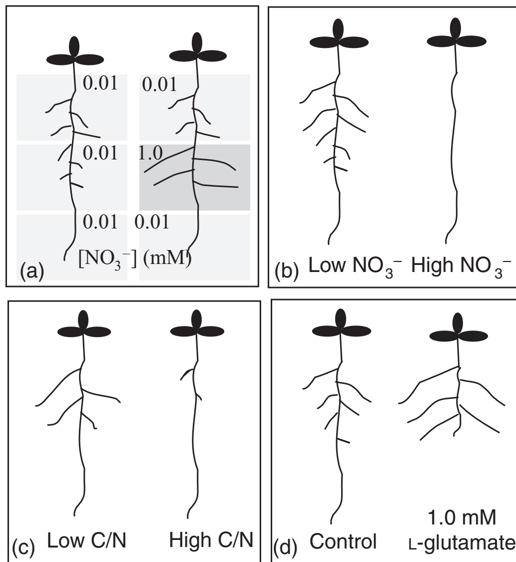

Figure 11.1 Diagrammatic illustrations of four characterized N-related morphological adaptations in *Arabidopsis* roots. (a) The localized stimulatory effect of NO_3^- on LR proliferation. (b) The systemic inhibitory effect of high NO_3^- on LR development. (c) The high C–N ratio-induced inhibition on LR initiation. (d) The effects of glutamate on the root system, including an inhibitory effect on primary root growth and a stimulatory effect on both LR initiation and LR development. Reproduced from Zhang *et al.* (2007), with permission.

of higher plants and occurs in many species and also in response to other nutrients (Robinson, 1994).

11.2.2 The systemic inhibitory effect of high NO_3^- on LR development

When *Arabidopsis* roots were exposed to a uniform high NO_3^- supply (above 10 mM), the number of visible LRs was significantly reduced (Zhang *et al.*, 1999) (Fig. 11.3). This response is believed to be due to at least partly the internal accumulation of the nitrate (NO_3^-) ion, as the *nia1nia2* mutant of *Arabidopsis*, which is defective in both the NADH-specific and NAD(P)H-bispecific forms of the enzyme nitrate reductase and therefore unable to convert NO_3^- to N metabolites, is found to be more sensitive to this high NO_3^- effect (Zhang *et al.*, 1999). When the root system of an *Arabidopsis* seedling is divided into two halves with only one half exposed to high N, the inhibitory effect occurs in the entire root, including the half that is not directly exposed to the high NO_3^- supply, which suggests that this inhibitory effect is systemic. It is believed that the signal is perceived in the shoots (Zhang *et al.*, 1999; Zhang & Forde, 2000). Primary root meristems appear to play a role in the high NO_3^--induced LR inhibition, as their removal abolishes this response (Zhang *et al.*, 2007).

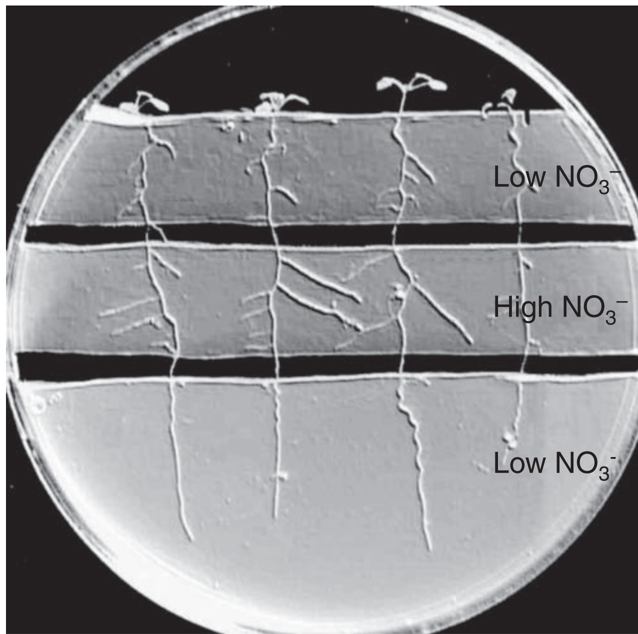

Figure 11.2 The localized stimulatory effect of NO_3^- in *Arabidopsis*. Shown are *Arabidopsis* seedlings grown on the surface of a segmented agar plate, with a localized high KNO_3 supply (1.0 mM) in the middle segment and a low NO_3^- supply (0.01 mM) in the top and bottom segments. Note the localized NO_3^- supply caused an increase in LR length in the middle segment.

11.2.3 The high C–N ratio-induced repression of LR initiation

This response occurs under a very specific condition, that is, when *Arabidopsis* seedlings are grown on media containing high sucrose and low N. Under this condition, LR initiation is dramatically suppressed, resulting in a reduction of the total number of LRs (including visible LRs and LR primordia). As reducing the C–N ratios, by either reducing the sucrose concentration while maintaining the low nitrogen concentration or increasing the N concentration while maintaining the high sucrose concentration, removes this effect and restores LR initiation, it has been proposed that the cause of the effect is the high C–N ratios, rather than the low N supply itself (Malamy & Ryan, 2001). The precise signals and the site of their perception are currently unknown.

11.2.4 The L-glutamate-induced regulation on root system architecture

When *Arabidopsis* seedlings were grown on media with low concentrations (0.05–0.5 mM) of L-glutamate, their root system was modified by a combination of three different effects: inhibition of primary root growth, stimulation

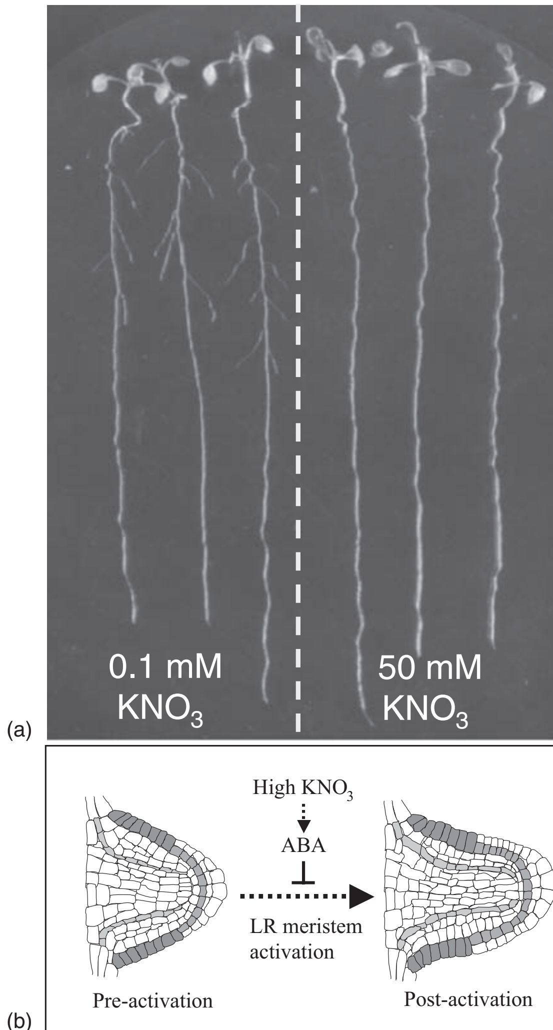

Figure 11.3 The systemic inhibitory effect of high NO_3^- on LR development. (a) The 10-day-old *Arabidopsis* seedlings grown on media with either 1.0 mM or 50 mM KNO_3. Note the seedlings on 50 mM KNO_3 display no visible LRs. (b) A diagram showing the specific stage of the high NO_3^--induced LR inhibition.

of the outgrowth of LRs behind the root apex and an inhibition of LR elongation (Walch-Liu *et al.*, 2006), resulting in a shorter, more branched root system which is similar to that observed with P deficiency (Linkohr *et al.*, 2002). The connection between the three different effects has not yet been well established, but it is possible that the inhibitions on both primary root growth and LR elongation are mediated by the same mechanism and that the stimulation of LR outgrowth is a secondary effect, which is caused by the inhibition of

primary root growth. This response was specific to L-glutamate, as treatment with similar or even higher concentrations of structurally related amino acids, such as aspartate, γ-aminobutyric acid (GABA), D-glutamate or glutamine, did not induce a similar response. This inhibitory effect on primary roots requires a direct exposure of the root tips to L-glutamate, which suggests that the signal is perceived at the primary root tip (Walch-Liu & Forde, 2008).

The existence of different forms of N-related morphological adaptations in *Arabidopsis* roots provides a good illustration of the complexity and sophistication of the adaptive strategies of higher plants to N. For example, these adaptations show that *Arabidopsis* roots can respond to a variety of N signals, which include the different forms of N (NO_3^- and L-glutamate), their quantity, spatial distribution (Robinson, 1994; Zhang & Forde, 1998; Zhang *et al.*, 1999) or relative abundance in comparison with other nutrients (Malamy & Ryan, 2001). They also demonstrate that *Arabidopsis* roots display different morphological responses to the same N form, such as the dual responses of LRs to NO_3^- (the localized stimulation of LR elongation and the systemic inhibition of LR development) (Zhang *et al.*, 1999) and the different responses of primary roots to low NO_3^- observed by Remans *et al.* (2006b), who found that when *Arabidopsis* seedlings were transferred from a high NO_3^- (10 mM KNO_3) to a low NO_3^- (0.5 mM KNO_3) supply, the low NO_3^- supply could have either a stimulatory or an inhibitory effect on LR development in different parts of the primary roots depending on the history of their previous NO_3^- exposure. In part of the primary roots that had a previous high N exposure, the low NO_3^- supply stimulated LR development. On the other hand, the low NO_3^- supply suppressed LR formation in part of the primary roots that had no such previous NO_3^- exposure. Another example of the complexity of the N adaptive strategies in *Arabidopsis* roots is that the different morphological adaptations occur at different stages of LR development (see Section 11.3 for details), which indicates that N signals control multiple steps in LR development. Further complexity is added by variations among different *Arabidopsis* ecotypes in their sensitivity to N (NO_3^- and L-glutamate) signals and interactions between different N signals in controlling the same developmental events (Walch-Liu & Forde, 2008). The complexity of the N-related morphological adaptations in *Arabidopsis* roots suggests that there are different N-sensing mechanisms involved in root development.

11.3 The developmental context of N-related morphological adaptations in *Arabidopsis* roots

From the developmental perspective, it is interesting that all the above-mentioned morphological adaptations in *Arabidopsis* roots involve LR development. This is not surprising considering that LRs make up the largest constituent of the root system of a plant and their number varies significantly between individual plants. In addition, LRs are considered to be far

more effective than the primary root in exploration of a larger soil volume (Robinson, 1994). Such characteristics make LR development an ideal target for morphological modifications. In *Arabidopsis*, LRs are derived from a specific cell layer, the pericycle, and their development has been well characterized morphologically (Malamy & Benfey, 1997; Casimiro *et al.*, 2003) and consists of a number of morphologically easily identifiable stages, such as initiation, primordium development, emergence, meristem activation and outgrowth. Interestingly, different N-related morphological adaptations take place at different stages of LR development. For example, the high C–N ratio-induced LR repression affects mainly the initiation step and causes a reduction in the total number of LRs (Malamy & Ryan, 2001). The systemic inhibitory effect controls the activation of newly formed LR meristems (Zhang *et al.*, 1999; De Smet *et al.*, 2003) and, under some conditions, also the emergence of LR primordia (Tranbarger *et al.*, 2003). The localized stimulatory effect mainly controls LR outgrowth (Zhang & Forde, 1998; Zhang *et al.*, 1999). The different developmental contexts of the N adaptations in *Arabidopsis* roots are important keys for separating different adaptive mechanisms/pathways. It is important to point out that some N-related adaptations also involve primary roots. For example, the adaptation to L-glutamate includes an inhibition of primary root growth. There is also evidence that NO_3^- stimulates the growth of both LRs and primary roots (Walch-Liu *et al.*, 2006; Walch-Liu & Forde, 2008).

11.4 Mechanisms of N-related morphological adaptations in *Arabidopsis* roots

In addition to morphological characterization, significant progress has also been made in the past decade in understanding the mechanisms of the different N-related morphological adaptations of *Arabidopsis* roots. In this section, we will consider such progresses in each of the adaptations.

11.4.1 Mechanisms of the localized stimulatory effect of NO_3^- on LRs

Several important advances have been made in the past decade to dissect the mechanisms involved in this adaptive response. One such advance is the establishment of the exact role of NO_3^- in it. In the 1970s and 1980s, it was believed that this morphological adaptation was due to a nutritional effect; that is, the enhanced LR proliferation in the NO_3^--rich zone was caused either by the increased N nutrition itself or by an increased flux of growth-stimulating substances such as carbohydrates or hormones as a direct result of an increased N nutrition (Hackett, 1972; Drew *et al.*, 1973; Granato & Raper, 1989; Sattelmacher *et al.*, 1993). However, experimental evidence obtained from *Arabidopsis* indicates that this explanation is incorrect and that NO_3^- acts

as a signal rather than a nutrient in this adaptive response (Zhang & Forde, 1998, 2000; Zhang *et al.*, 1999; Remans *et al.*, 2006a). Supporting evidence for this conclusion includes:

1. An NR-deficient *Arabidopsis* mutant, with a much reduced capacity to assimilate NO_3^-, is able to respond normally to a localized NO_3^- supply.
2. Localized applications of other N sources, such as NH_4^+ and glutamine, do not stimulate LR elongation.

The most significant breakthrough is the identification of a key regulatory component of the regulatory pathway involved in this response, ANR1, which is an MADS box transcription factor (Zhang & Forde, 1998). Transgenic plants with reduced ANR1 expression, either by antisense or by co-suppression, showed a significant reduced sensitivity to this NO_3^--induced morphological response. This finding provides further support for the role of NO_3^- as a signal in the localized stimulatory effect and also strong evidence for the existence of a specific signalling pathway for this adaptive response in *Arabidopsis*. A further important finding is that a putative NO_3^- transporter, AtNRT1.1 (also known as CHL1; Tsay *et al.*, 1993), plays a key role in this response and probably acts upstream of *ANR1* (Remans *et al.*, 2006a). AtNRT1.1 is a dual-affinity NO_3^- transporter with NO_3^- uptake activities at both low and high external NO_3^- concentrations (Tsay *et al.*, 1993; Wang *et al.*, 1998; Liu *et al.*, 1999). AtNRT1.1-defective mutants, like the ANR1 down-regulated plants, have a significantly reduced sensitivity to the localized stimulatory effect of NO_3^- on LR elongation (Remans *et al.*, 2006a). This phenotype is not due to lower NO_3^- uptake activity in the mutants and is not suppressed when the NO_3^--rich patch is supplemented with an alternative N source. Most significantly, this reduced sensitivity to NO_3^- was accompanied by reduced ANR1 transcript abundance, suggesting that AtNTR1.1 may play a direct role in transcriptional regulation of *ANR1*. Thus, a short regulatory circuit has been established for the localized stimulatory effect, which includes a putative NO_3^- sensor AtNRT1.1 and a downstream transcription factor ANR1.

There are still important questions to be answered about the mechanism of this morphological response. Firstly, the exact role of AtNRT1.1 has not yet been established. It has been suggested that AtNRT1.1 may act as an NO_3^- sensor to detect external NO_3^- availability. Although there is increasing supporting evidence for such a role for several putative transporters, direct experimental evidence for this specific AtNRT1.1 function (as an NO_3^- sensor) is still lacking. Secondly, it is currently unclear how AtNRT1.1 regulates *ANR1* expression. Thirdly, mechanisms about how *ANR1* acts to control LR elongation have not yet been established. Lastly, it is not known whether the regulatory pathway identified in *Arabidopsis* is conserved in other plant species.

There is some evidence that phospholipid signalling may be involved in the localized stimulatory effect of NO_3^- on LRs. For example, *Arabidopsis* seedlings that overexpress *PLDε*, one of the 12 *arabidopsis* genes encoding

phospholipase D which catalyzes the hydrolysis of phosphatidylcholine to form phosphatidic acid, show more elongation of LRs than wild-type (WT) plants at low rates of nitrate supply (Hong *et al.*, 2009). Conversely, seedings in which the *PLDε* gene is knocked out show reduced LR elongation. When seedlings were transferred from high to low nitrate supply, expression of *AtNRT1.1* was higher in the plants overexpressing *PLDε* than those with the gene knocked out and the wild types. It seems likely that phospholipase D, and the lipid messenger phosphatidic acid that its action gives rise to, is involved in linking the signalling of N status to the morphological response of plants.

11.4.2 Mechanisms for the systemic inhibitory effect of high NO_3^- on LR development

As mentioned previously, there is experimental evidence that the signal responsible for the high NO_3^--induced inhibition of LR development is perceived in the shoots (Zhang *et al.*, 1999; Forde, 2002). This raises the question of how the signal is passed on from the shoots to the roots. Walch-Liu *et al.* (2006) examined the possible role of auxin in this long-distance signalling process and found that *Arabidopsis* seedlings grown on high (50 mM) NO_3^- had significant lower indole-3-acetic acid (IAA) content in the shoots compared with those on low (1.0 mM) NO_3^-. A similar observation has also been reported in soybean (Caba *et al.*, 2000), where it was found that plants grown on 8 mM KNO_3 had fourfold less IAA in the root tissues than plants grown on 1 mM KNO_3. These results show that high NO_3^- accumulation in the shoots can cause a reduction in auxin content in the roots. It is possible that the reduced auxin supply in the roots could be the cause of the high NO_3^--induced LR inhibition. However, observation that exogenously applied auxin could not overcome the inhibitory effect of high NO_3^- in *Arabidopsis* seedlings (Zhang *et al.*, 2007) argues against this hypothesis and indicates that the observed reduction in auxin content is either independent of the inhibitory effect, or that external auxin is unable to substitute for auxin delivered via the phloem.

There are several lines of evidence supporting a role of another hormone, abscisic acid (ABA), in the high NO_3^--induced LR inhibition (Signora *et al.*, 2001). First, the high NO_3^--induced LR inhibition was significantly reduced in several ABA-deficient (*aba1-1*, *aba2-3*, *aba2-4*, and *aba3-2*) and ABA-insensitive mutants (*abi4-1*, *abi4-2*, and *abi5-1*) (Signora *et al.*, 2001). Second, exogenously supplied ABA mimics the effect of high NO_3^- (De Smet *et al.*, 2003) on LR development. Third, mutants that are identified based on their insensitivity to the inhibition of LRs by ABA (designated as *labi*, for *lateral root ABA insensitive*) also showed a reduced sensitivity to the high NO_3^--induced LR inhibition (Zhang *et al.*, 2007).

Malamy (2005) raised the possibility that the high NO_3^--induced inhibition of LR development could be caused by an osmotic effect. This argument is based on the observations that high osmotic potential repressed LR

development and mimicked the inhibitory effect of high NO_3^- and that this osmotic repression also involved ABA (Deak & Malamy, 2005). However, a distinct difference exists between the LR repression caused by osmotic potential and the inhibition induced by high NO_3^-/ABA as osmotic repression can be overridden by auxin (Deak & Malamy, 2005), whilst the inhibition by high NO_3^-/ABA is auxin-independent (Signora *et al.*, 2001; De Smet *et al.*, 2003). This difference suggests that the two effects, although morphologically similar, are probably not regulated by the same mechanism. It is possible that high NO_3^- could affect LR development through both osmotic repression (which can be overridden by auxin) and osmotic-independent, NO_3^-/ABA-specific inhibition (which is not auxin reversible).

A report suggests that soil bacteria may affect the inhibitory effect of high NO_3^- on LR development. It was found that this inhibitory effect was significantly reduced in the presence of a plant growth-promoting rhizobacterium (PGPR), *Phyllobacterium* strain STM196 (Mantelin *et al.*, 2006), and this impairment was accompanied by an alteration in the expression of a number of NO_3^- transporter genes including *AtNRT1.1*, *AtNRT2.1*, *AtNRT2.5*, and *AtNRT2.6*, indicating a general interaction between rhizobacterium and NO_3^- signalling. This observation suggests that morphological adaptation of the root system to N integrates other environmental signals.

11.4.3 Mechanisms for the high C–N ratio-induced LR inhibition

Important knowledge has also been obtained about the mechanisms of the high C–N ratio-induced LR repression. First, there is evidence that this response is linked to auxin, which is a key regulator of LR initiation (Casimiro *et al.*, 2003). The high C–N ratio-induced repression was found to be accompanied by enhanced auxin accumulation in the hypocotyls (Malamy & Ryan, 2001), which suggests that this effect is probably due to a reduced shoot-to-root auxin transport. As LR initiation depends on the auxin level, a reduced auxin supply in the roots can lead to a repression of this developmental process. The most important piece of information about the mechanisms of the high C–N ratio-induced LR repression comes from the characterization of an *Arabidopsis* mutant, *lin1* (for *lateral root initiation 1*), that overcomes this repression and produces a highly branched root system on media with high sucrose-to-nitrogen ratios (Malamy & Ryan, 2001). The *lin1* was found to carry a missense mutation in a putative high-affinity NO_3^- transporter gene, *AtNRT2.1* (Little *et al.*, 2005). A direct link between *AtNRT2.1* defect and *lin1* phenotype was confirmed by both complementation and the observation that allelic mutants of *lin1*, including one in which the *AtNRT2.1* gene is completely deleted, showed similar phenotypes to *lin1* on high C–N media. In addition, *linl* plants, like other *AtNRT2.1*-defective mutants, had reduced NO_3^- uptake and NO_3^- content (Little *et al.*, 2005). Interestingly, LR initiation in *lin1* is increased relative to WT even when seedlings are grown on NO_3^--free media, suggesting that the mutant phenotype is NO_3^--independent.

This latter observation has led to the hypothesis that AtNRT1.1 may act as an NO_3^- sensor or signal transducer in this morphological response (Little *et al.*, 2005).

Important gaps exist in our knowledge about the mechanisms of the high C–N ratio-induced LR repression. First, it is not clear about the exact signal of the response. Originally, it was thought that the high C–N ratio, not low N or NO_3^-, was the signal. However, following the identification of LIN1 as AtNRT2.1, it was suggested that AtNRT2.1 could act as an NO_3^- sensor, which implies that NO_3^- is at least part of the signal. This confusion must be resolved to have a clear understanding of the true mechanism of this response. Second, there is also little understanding of how AtNRT2.1 acts to repress LR initiation. Little *et al.* (2005) hypothesized that AtNRT2.1 was a repressor of LR initiation, but how it actually functions in such a role at either molecular or cellular level has not yet been established.

In a recent comparison of 12-day-old *Arabidopsis* seedlings grown on Murashige and Skoog medium containing either 12 mM nitrogen with 1% sucrose or 60 mM nitrogen with 1% sucrose, there were fewer LR primordia per unit length of primary root in the high C–N medium than in the low C–N medium (although not a statistically significant difference), but there were significantly more LR primordia in the high C–N treatment than in the low C–N treatment when these were counted per 100 cortical cells (Dubrovsky *et al.*, 2009). A large part of the response to the high C–N ratio appears to give significantly larger cortical cells.

11.4.4 Mechanisms for the regulatory roles of glutamate on RSA

In animals, glutamate acts as a neurotransmitter, and this function requires specific receptors, known as glutamate receptors (GLRs). Homologues of GLRs exist in plants. In *Arabidopsis*, there are 20 *GLR* genes, which are expressed collectively throughout the plant (Chiu *et al.*, 2002). There is some discrepancy in the literature whether the morphological adaptation of *Arabidopsis* roots to glutamate involves GLRs. Sivaguru *et al.* (2003) reported that glutamate-induced inhibition of root growth could be blocked by a specific antagonist of mammalian ionotropic GLRs, 2-amino-5-phosphonopentanoate, which suggests that GLRs are required for this glutamate regulation. However, this GLR function was not found in another study (Walch-Liu *et al.*, 2006) in which three GLRs antagonists, including 6,7-dinitroquinoxaline-2,3-dione (DNQX), MK801 and 2-amino-5-phosphonopentanoate, and a cycad-derived agonist of mammalian GLRs, β-methylamino-L-alanine (BMAA), were used together with L-glutamate, and none of the antagonists or agonist was found to affect the L-glutamate-induced regulation of RSA in *Arabidopsis* seedlings (Walch-Liu *et al.*, 2006).

There is some evidence that the regulatory role of L-glutamate in *Arabidopsis* roots is linked to auxin. The *aux1-7* mutant, which is defective in the auxin efflux carrier AUX1 (Bennett *et al.*, 1996), had reduced L-glutamate sensitivity,

suggesting a possible interaction between L-glutamate and auxin signalling. However, two loss-of-function mutants at the *AXR1* locus (*axr1-3* and *axr1-12*) were hypersensitive to L-glutamate, indicating the complex interplay between the glutamate response and auxin signalling.

Interestingly, NO_3^- (0.5 mM) antagonizes the inhibitory effect of L-glutamate on primary root growth. When *Arabidopsis* seedlings were grown in the presence of relatively high concentration of NO_3^- (0.5 mM), they lost their sensitivity to L-glutamate in respect to this inhibitory effect (Walch-Liu & Forde, 2008). Several lines of evidence show that this antagonism requires the putative NO_3^- transporter AtNRT1.1. First, an *AtNRT1.1*-defective mutant (*chl1-5*) failed to show this antagonism and was sensitive to the Glu-induced inhibition of primary root growth (Walch-Liu & Forde, 2008). Second, the lost NO_3^- sensitivity in the *AtNRT1.1* mutant was restored by constitutive expression of the wild-type version of AtNRT1.1, but not by the expression of the non-phosphorylatable version of this protein (Walch-Liu & Forde, 2008). Since non-phosphorylatable AtNRT1.1 retains most of its NO_3^- transport activities (Liu & Tsay, 2003), the above observation suggests that AtNRT1.1 plays a regulatory role, rather than NO_3^- transport role, in the glutamate-induced regulation of RSA.

11.5 Role of NO_3^- transporters in N-related morphological adaptations

A surprising discovery from studying the N-related morphological adaptations in *Arabidopsis* roots is the role of putative NO_3^- transporters in these responses. For example, the previously known dual-affinity NO_3^- transporter AtNRT1.1 has been found to play a role in both the stimulatory effect of NO_3^- on LRs and the antagonistic interaction between NO_3^- and L-glutamate in controlling primary root growth (Remans *et al.*, 2006b; Walch-Liu & Forde, 2008). In addition, a second NO_3^- transporter, the high-affinity NO_3^- transporter AtNRT2.1, is involved in the adaptation of *Arabidopsis* roots to high C–N ratios (Little *et al.*, 2005). Most importantly, in all the above-mentioned three cases, these transporters appear to be playing a regulatory role, rather than the role of NO_3^- transporters. For example, Remans *et al.* (2006a) showed that NRT1.1-defective mutants displayed a strongly decreased root proliferation in NO_3^--rich patches and that this phenotype could not be restored by supplying an alternative N source. This indicates that AtNRT1.1 plays a regulatory role in this response that is independent of its role as an NO_3^- transporter. A similar conclusion was reached regarding the role of AtNRT1.1 in the NO_3^--glutamate antagonism in the regulation of primary root growth (Walch-Liu & Forde, 2008) and also about the role of AtNRT2.1 in the response to high C–N ratios (Little *et al.*, 2005) (see previous discussion in this chapter). These observations led to the hypothesis that the NO_3^- transporters may act

as NO_3^- sensors (Little *et al.*, 2005; Remans *et al.*, 2006a, 2006b; Walch-Liu & Forde, 2008; Forde & Walch-Liu, 2009). This hypothesis is consistent with a number of other observations (Forde & Walch-Liu, 2009) and is further supported by the recent findings that a *nrt1.1* mutant is captured in a genetic screen for NO_3^- regulatory mutants and that *nrt1.1* mutants are impaired in NO_3^- regulation of gene expression over a wide variation of NO_3^- concentrations (Wang *et al.*, 2009). These authors used a genetic screen that employed an NO_3^--inducible promoter fused to the yellow fluorescent protein marker gene YFP for NO_3^- regulatory mutants and, among the mutants obtained, they found one (*nrg1*) carrying a mutation in *AtNRT1.1*. The *nrg1* mutation disrupted NO_3^- regulation of a number of NO_3^--responsive genes including *NIA1*, *NiR* and *NRT2.1*, and this regulatory defect is not explained by reduced NO_3^- uptake (Wang *et al.*, 2009).

There is ample evidence in other organisms such as animals and yeasts that transporter proteins can also act as nutrient sensors (Holsbeeks *et al.*, 2004). For example, in the yeast *Saccharomyces cerevisiae*, two phosphate carriers, Pho84 and Pho87, are involved in the sensing of a phosphate signal in the activation of protein kinase A pathway (Giots *et al.*, 2003). It has also been reported that the general amino acid permease Gap1 (X52633) in yeast also functions as an amino acid sensor (Donaton *et al.*, 2003).

11.6 Biological significance of the localized stimulatory effect

Of the above-mentioned N-related morphological adaptations in *Arabidopsis* roots, the systemic inhibitory effect of high NO_3^- on LR development is the one with the clearest biological relevance. It is well established that plants can alter the relative growth of roots and shoots according to the level of nutrient supply. Under nutrient-limiting conditions, root growth is favoured resulting in a reduction of the shoot–root ratio, while when nutrient supply is abundant, shoot growth is favoured, which results in an increase in shoot–root ratio. Therefore, plants are expected to restrict root growth under high N supply, and the systemic inhibitory effect of high NO_3^- on LR development provides an effective mechanism for plants to do so.

There have been questions whether it is beneficial for plants to grow more LRs in NO_3^--rich patches (Robinson, 1996). This is because the NO_3^- is easily diffusible in soil and morphological changes such as LR growth are relatively slow. However, Hodge *et al.* (1999) and Robinson *et al.* (1999) showed that, when two perennial grasses (*Poa pratensis* and *Lolium perenne*) with different capacities to respond to the stimulatory effect of NO_3^- were grown on the same patch with NO_3^- as the N source, the species that was more responsive to NO_3^- (*L. perenne*) produced more roots in the patch and captured more N from it in comparison with the other species (*P. pratensis*). This strong

correlation between root length density and NO_3^- capture in both species emphasized the functional significance of the localized stimulatory effect of NO_3^- and demonstrated that, even with a mobile nutrient such as NO_3^-, this response still offers plants a competitive advantage in resource capture over their neighbouring plants (Hodge *et al.*, 1999; Robinson *et al.*, 1999).

The biological significance of the other two N-related morphological adaptations, namely, the high C–N ratio-induced LR repression and L-glutamate-induced regulation of RSA, is currently unclear. It has been speculated that the high C–N ratio-induced suppression of LR initiation may reflect a stress response mechanism by which plants respond to high C–N ratios as if they are stress signals and therefore limit root growth as a part of stress responses (Little *et al.*, 2005). However, this interpretation does not explain why this response is specific to C–N ratios (for example, low C and low N would be stronger stress signals) and why increasing both C and N while maintaining the same C–N ratios also induces the same response. The glutamate response could be interpreted as a mechanism by which plants respond to variations in the external concentration of amino acids (Forde & Waltch-Liu, 2009), which can represent a significant proportion of the dissolved N fraction in soils and also a significant source of N for plant nutrition (Miller & Cramer, 2005). It has also been further suggested that the response to glutamate may reflect a foraging mechanism by which plants use a specific amino acid such as glutamate to locate an organic N source and to use such information to modify their root growth in order to explore such N sources (Forde & Walch-Liu, 2009). However, if that is true and glutamate merely serves as a signal for a mixed N source, it raises the question of why plants have to respond so specifically to this amino acid but not to other amino acids, which can also serve as signals of an organic N source.

11.7 Concluding remarks

Considerable progress has been made over the past decade in both characterizing N-related morphological adaptations in the root system of *Arabidopsis* plants and in understanding the cellular and molecular processes underlying such adaptations. Figure 11.4 provides a summary of our current understanding of the field. Studies of such adaptations reveal insights of novel regulatory properties of nitrate transporters in N sensing and root development.

The future challenges are to understand the mechanisms of the N-related morphological adaptations and to establish whether the adaptations found in *Arabidopsis* and also their regulatory mechanisms are conserved in other plants and also the biological significance of the adaptations. *Arabidopsis* has a root system typical of those found in eudicots, and is a suitable model for plants other than monocots. In a study on another species frequently studied in respect to the effect of nitrogen on root system development, *Nicotiana*

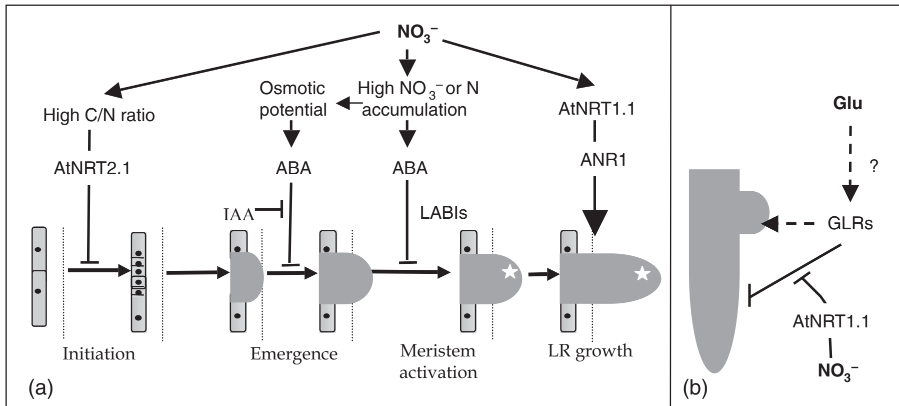

Figure 11.4 A summary of our current understanding of the four N-related morphological adaptations in *Arabidopsis* roots and their regulatory mechanisms. (a) Adaptations linked to LR development, including the repression of LR initiation by high C–N ratios, the inhibition of LR meristem activation by high nitrate and the stimulatory effect of locally applied nitrate on LR growth. The possible overlap between the inhibitory effects of high nitrate and osmotic stress is also indicated. (b) The effect of glutamate on root morphology and the antagonism between nitrate and L-glutamate. Modified from Zhang *et al.* (2007), with permission.

sylvestris, wild-type plants were shown to have LR production strongly stimulated by supply of nitrate, up to an optimum concentration of 1–5 mM (Pellny *et al*., 2008). Furthermore, like *Arabidopsis*, these plants showed antagonism of LR production by high nitrate, an antagonism that was reduced by supply of sucrose. However, these plants differed from *Arabidopsis* in that NH_4NO_3 stimulated LR production at lower concentrations than KNO_3, indicating a role for ammonium in LR development. As well as the need to fully understand the mechanisms of the responses of *Arabidopsis* root systems to nitrogen, there are fascinating differences between plant species to be exposed.

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Chapter 12

MITOCHONDRIAL REDOX STATE, NITROGEN METABOLISM AND SIGNALLING

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Abstract: Nitrogen is often a limiting factor for plant growth and development, and therefore there is considerable interest and potential agronomic benefit not only in understanding the mechanisms that determine nitrogen use efficiency (NUE) but also in identifying new targets for NUE improvement. Much attention in recent years has focused on the core pathways and enzymes involved in primary nitrogen assimilation as well as the processes of N uptake and transport. However, recent studies on mitochondrial Complex I mutants indicate that enzymes involved in energy metabolism in the mitochondria could also be useful targets for improving NUE. The *Nicotiana sylvestris* cytoplasmic male sterile II (CMSII) mutant lacks respiratory Complex I, but high rates of respiration are sustained because of the presence of alternative NADH dehydrogenases. The CMSII mutation has striking effects on leaf amino acids, which are the focal point around which primary N assimilation and associated carbon metabolism and photorespiration and export occur, and on pyridine nucleotides, which are essential for metabolism and signalling. The absence of major mitochondrial NADH dehydrogenase (Complex I) in the CMSII mutant results in an N-rich phenotype and altered N signalling. Adjustments in the abundance of Complex I relative to the alternative NADH dehydrogenases could therefore provide a mechanism for improved NUE.

Keywords: amines–nitrate ratios; amino acids; C–N signalling; mitochondria; NADH dehydrogenases; photorespiration; photosynthesis; pyridine nucleotides; respiration; root architecture; shoot–root ratios

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12.1 Introduction

Plants devote a significant proportion of their carbon and energy budgets to the uptake and assimilation of N, which is a major constituent of many important plant components such as amino acids, proteins, nucleic acids and co-factors. Nitrogen is required in greater quantities by plants than any other mineral because of the abundant N invested in the photosynthetic machinery. Photosynthesis uses carbon dioxide, water and inorganic N to produce sugars, organic acids, amino acids and a whole range of secondary compounds. Nitrogen deficiency promotes root growth with an increased number of lateral roots, but it has negative impacts on shoot growth and productivity, accelerating leaf senescence and triggering an early transition from vegetative growth to flowering (Scheible *et al.*, 2004). Numerous studies have shown that carbon and nitrogen metabolites are monitored by the cell and act together to orchestrate gene expression, thus determining transcriptome profiles that are appropriate to nutritional and metabolic status (Wang *et al.*, 2000; Palenchar *et al.*, 2004). Post-translational controls are also important with the phosphorylation of proteins such as phosphoenolpyruvate carboxylase and nitrate reductase (NR) (Van Quy *et al.*, 1991; Kaiser & Huber, 1994), allowing regulation of enzyme activities by metabolite effectors (Moraes & Plaxton, 2000; Smith *et al.*, 2000).

Whole plant C–N interactions are highly complex with multiple sites of reciprocal control. For example, N signals participate in the regulation of genes involved in multiple pathways including photosynthesis, chlorophyll and plastid protein synthesis, phenylpropanoid and flavonoid metabolism (Scheible *et al.*, 2004; Diaz *et al.*, 2006). N signals also interface with a wide range of other environmental, metabolic and developmental signals that together determine overall plant growth and performance. Interpathway flux controls and metabolite cross-talk in the orchestration of gene expression follow simple basic principles of supply and demand that ensure an appropriate plant C–N balance relative to developmental and metabolic controls. Substrates, metabolites and products are important in the control of the expression and activity of key enzymes that allows coordination of C and N assimilation, as they act as 'sensors' of C–N status.

The C–N interaction in individual cells takes place within a context of energy use and production and involves cooperation between different subcellular compartments (Foyer *et al.*, 1994a, 1994b; Noctor & Foyer, 1998; Gardeström *et al.*, 2002; Foyer *et al.*, 2003). Plants take up and assimilate various forms of N, although nitrate is generally the principal form available to the roots of crop plants. Nitrogen is acquired from the soil through uptake of nitrate and ammonium catalysed by a range of low- and high-affinity transport systems. Nitrate assimilation requires reductant to be supplied to both NR and nitrite reductase. However, despite extensive analysis of the regulation of NR expression and activity, it has not yet been established which factors are most important in limiting nitrate reduction in leaves (Foyer *et al.*, 1994a, 1994b; Lejay *et al.*, 1997; Kaiser *et al.*, 2002). Given that the K_mNADH

of NR may be several-fold higher than the cytosolic concentration of the reduced form of nicotinamide adenine dinucleotide, the ability of chloroplasts or mitochondria to deliver reductant to the cytosol could be the key in determining the rate of nitrogen assimilation (Krömer & Heldt, 1991; Hanning & Heldt, 1993). Low photosynthesis rates limit NR activity through decreased reductant availability and through effects on post-translational inactivation (Kaiser *et al.*, 2000, 2002). Nitrate reduction is also favoured by high rates of photorespiration (Rachmilevitch *et al.*, 2004), which involves complex redox cycling with reductant originating in the chloroplasts and mitochondria exported to the cytosol and peroxisomes (Hanning & Heldt, 1993).

The formation of organic acids required to support amino acid production may also be subject to redox modulation. Compounds such as 2-oxoglutarate (2-OG) act as carbon skeletons for amino acid synthesis, and their production requires oxidation through respiratory pathways involving the cytosol and mitochondria (Smith *et al.*, 2000; Hodges, 2002; Foyer *et al.*, 2003). Amino acid synthesis requires that nitrate reduction occurs alongside carbon oxidation, and thus, cellular redox status may be an important factor integrating the two processes. The assimilation of nitrate rather than ammonia may serve to decrease mitochondrial respiration rates in the light (Cousins & Bloom, 2004). Moreover, nitrate reduction is significantly stimulated by anoxia. Nitrate reduction and mitochondrial electron transport can therefore compete for reductant under some conditions. Genetic modification of mitochondrial respiration may therefore hold promise for improving nitrogen assimilation. Mitochondrial pathways produce the carbon skeletons required for ammonia assimilation, but the significance of these events in the overall regulation of N assimilation remains uncertain (Hodges, 2002; Foyer *et al.*, 2003). Mitochondrial metabolism and electron transport have been extensively analysed (e.g. Vanlerberghe *et al.*, 2002; Michalecka *et al.*, 2003; Moore *et al.*, 2003; Fernie *et al.*, 2004a, 2004b; Rasmusson *et al.*, 2004), but there are relatively a few reports on the potential influence of the leaf mitochondrial electron transport chain over the integration of major leaf metabolic pathways. The accumulating evidence of altered C–N metabolism and signalling in the CMSII mutant, especially as nitrogen enrichment is associated with the transition from NADH oxidation by Complex I to the alternative dehydrogenases, therefore represents an important advance in our understanding of how mitochondrial processes influence N metabolism. Moreover, recent reports on similar Complex I mutants in other species, such as *Arabidopsis*, suggest that N enrichment is a general feature of altered mitochondrial redox metabolism that causes alterations in pyridine nucleotide metabolism.

12.2 The *Nicotiana sylvestris* mitochondrial cytoplasmic male sterile II mutant

The *N. sylvestris* CMSII mutant was isolated in the laboratory of Rosine De Paepe in Orsay, France (Gutierres *et al.*, 1997; Sabar *et al.*, 2000). It is one of a very few available stable homoplastic mutations that results in the

disappearance of a major respiratory complex from the mitochondrial electron transport chain. CMSII, which carries a mutation in the mitochondrial *nad7* gene and as a consequence completely lacks respiratory Complex I (Gutierres *et al.*, 1997; Pineau *et al.*, 2005), has proved to be a valuable tool in the analysis of how mitochondrial redox controls influence plant biology. The CMSII plants grow more slowly than the wild type (WT), but they remain viable because respiration is sustained through alternative mitochondrial NADH dehydrogenases (Sabar *et al.*, 2000). Respiration using only low-affinity NADH alternative mitochondrial dehydrogenases causes adjustments in the respiratory pathways that lead to a profound change in leaf NAD(H) availability and to significant increases in stress tolerance (Dutilleul *et al.*, 2003). The CMSII-dependent increases in the availability of this pyridine nucleotide have a critical influence on the rate of nitrate assimilation. The absence of Complex I in CMSII leaves is associated with increases in both NADH and NAD^+ (Dutilleul *et al.*, 2005). Taken together, results to date show that as a result of altered redox and energy metabolism, the modulation of respiratory electron transport chain in CMSII strongly impacts on primary metabolism with wide-ranging effects on respiration, photosynthesis, photorespiration, nitrogen assimilation, and C–N interactions (Sabar *et al.*, 2000; Dutilleul *et al.*, 2003, 2005; Priault *et al.*, 2006; Noctor *et al.*, 2007; Pellny *et al.*, 2008). Evidence concerning the impact of respiratory pathways on the plant C–N interaction, N signalling and on plant growth and development as revealed by studies in the CMSII mutant is discussed in the following sections.

12.2.1 Germination is delayed in the CMSII mutant compared to the wild type

Germination is usually designated as complete once the seed coat is ruptured by the radicle. It is initiated by water uptake and the resumption of metabolism and cell division, processes that require remobilization and/or assimilation of N to produce nucleotides and proteins. Nitric oxide (NO) is a potent regulator of seed germination in *Arabidopsis* (Bethke *et al.*, 2004, 2006a, 2006b; Libourel *et al.*, 2006). Nitrate can break seed dormancy in *Arabidopsis* (Bethke *et al.*, 2006b). However, the presence of nitrate did not appear to alter the germination of *N. sylvestris* seeds (Fig. 12.1). Germination was delayed in CMSII seeds compared to the WT under almost all conditions that were tested (Fig. 12.1). High KCl or N concentrations inhibited germination (Fig. 12.1). The sensitivity of the CMSII seeds to high KCl or N concentrations is perhaps surprising given the enhanced stress tolerance observed in CMSII plants (Dutilleul *et al.*, 2003).

12.3 Metabolite profiling in CMSII leaves reveals an N-rich phenotype

Metabolites and proteins define the metabolic signature that underpins phenotypic traits in plants. Metabolomics approaches aim to provide a

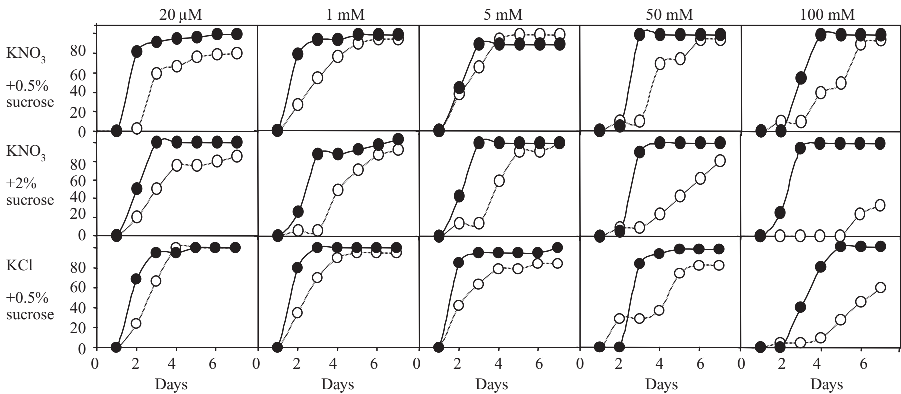

Figure 12.1 The effect of C and N availability on germination in WT (black circles) and CMSII (white circles) seeds. The frequency of germination (expressed here as a percentage) was measured on agar supplemented with different concentrations of potassium nitrate, potassium chloride and sucrose, as indicated on each panel.

comprehensive view of metabolite profiles, which provide a 'metabolic phenotype' even when genetic differences are not manifest in apparent effects on plant morphology and development (Weckwerth *et al.*, 2004). Unlike transcriptomics and, to a lesser degree, proteomics, metabolomics is confronted by analytical problems related to chemical heterogeneity (Fernie *et al.*, 2004a, 2004b). Thus, metabolite analyses have traditionally been targeted to a relatively small number of compounds within a given class defined by a particular functional group. No single technique is currently able to provide data on more than a fraction of the metabolites contained within plant cells, but metabolomics approaches can provide information on different classes of metabolites. Non-targeted approaches are made possible by the use of non-specific detectors such as mass spectrometry (MS) and nuclear magnetic resonance (Roessner *et al.*, 2000, 2001; Weckwerth *et al.*, 2004; Hirai *et al.*, 2005; Overy *et al.*, 2005; Mounet *et al.*, 2007). Gas chromatography (GC) electron-impact MS has rapidly become established as a technique of choice for the provision of information on a relatively large number of small metabolites because of its sensitivity and ease of reference to mass spectra fragmentation patterns in publicly available databases (Roessner *et al.*, 2000, 2001). Among the different types of MS detectors that can be coupled to GC, time-of-flight (TOF)-MS offers distinct advantages in profiling complex mixtures (Wagner *et al.*, 2003). Metabolite profiling by GC-MS thus provides a snapshot of many compounds involved in core pathways such as the tricarboxylic acid (TCA) cycle, nitrogen assimilation, amino acid synthesis, carbohydrate metabolism and photorespiration (Stitt & Fernie, 2003).

Targeted metabolite analysis and GC-MS-TOF approaches have demonstrated that the metabolic phenotype of CMSII leaves is characterized by a general increase in amino acids and organic acids and a decrease in starch (Dutilleul *et al.*, 2005). This metabolite profile is associated with high rates

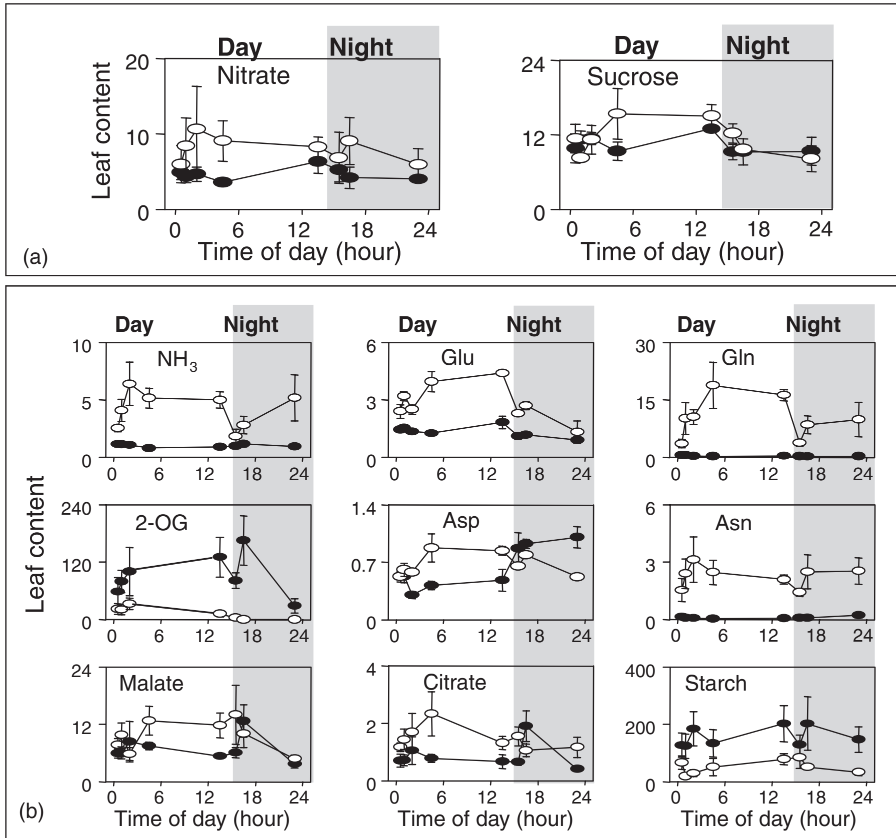

Figure 12.2 Day–night rhythms in metabolite contents and ratios are greatly modified in CMS leaves. Triplicate leaf samples were taken throughout a 14-hour day/10-hour night cycle. For citrate:2-OG, data indicate minimum values (2-OG was undetectable). Black circles, WT; white circles, CMSII. All values are in μmol/mg chlorophyll, except for 2-OG (nmol/mg chlorophyll) and ratios, which are mole/mole.

of nitrogen assimilation in the CMSII leaves together with an altered C–N balance (Rufty *et al.*, 1988; Scheible *et al.*, 1997; Dutilleul *et al.*, 2005) and altered N signalling (Pellny *et al.*, 2008). Nitrate assimilation rates are enhanced in the CMSII mutant, especially in the dark, and N-rich metabolites accumulate in CMSII leaves (Fig. 12.2; Dutilleul *et al.*, 2005). Marked increases in the leaf pools of organic acids such as citrate and malate are also observed (Fig. 12.2). The latter observation is consistent with the increased respiration rates observed in the CMSII mutant (Sabar *et al.*, 2000; Dutilleul *et al.*, 2003, 2005). Taken together, these data show that the switch from Complex I to the alternative dehydrogenases in the CMSII mutant results in a nitrogen-rich phenotype.

Altered NADH accumulation and partitioning in the mutant leaves has effects on nitrate assimilation and TCA cycle metabolites, and these act in concert to perturb the day/night coordination of C and N metabolism (Fig. 12.2). Leaf ammonia levels decreased rapidly on darkening in CMSII as did the Gln:Glu ratios, but these changes were followed by slow increases as the dark period progressed. Such effects are consistent with the inability of the mutant to shut down nitrate assimilation in the dark (Dutilleul *et al.*, 2005), a feature that is likely to be exacerbated by low 2-OG availability (Fig. 12.2). Day/night rhythms in enzyme activities and transcripts, as well as in metabolites involved in the C–N interaction, have been extensively characterized in *N. tabacum*. Such day/night rhythms can be explained by the lack of temporal coordination in supply and demand of key compounds such as 2-OG.

In marked contrast to the increases in leaf total free amino acid contents observed in the CMSII mutant leaves when plants are grown under optimal N availability, CMSII has similar amounts of leaf amino acids to the WT when plant growth with limiting N prevents (Pellny *et al.*, 2008; Hager *et al.*, 2010). Thus, sufficient nitrogen nutrition is required to allow expression of the enriched amino acid phenotype in CMSII. This observation is intriguing and might be explained by differences in the impact of the various factors regulating nitrogen nutrition at low and optimal N (Pellny *et al.*, 2008; Hager *et al.*, 2010). For example, the distribution of the control of the NR reaction by nitrate and by NADH availability might be different at low and high N, via effects of cytosolic nitrate concentrations directly or by regulation of NR expression. However, as discussed below, it may also be significant that NAD(H) pools were not increased in the CMSII leaves relative to the WT when plants were grown under conditions of low N nutrition.

12.4 Mitochondrial redox cycling is a key player in determining the rate of nitrate assimilation

In addition to integration with respiratory C flow, N assimilation in the leaves of C_3 species is also intimately associated with photorespiratory C flow, an interaction that further complicates the photosynthetic C and N interaction (Foyer *et al.*, 2006). Under most conditions, the photorespiratory cycle is much more rapid than primary nitrate reduction. Photorespiration can influence N assimilation in several ways (Foyer *et al.*, 2009). Ammonia liberated during the conversion of Gly to Ser in leaves is recycled through Gln and Glu (Keys *et al.*, 1978). Ammonia recycling probably occurs through the same glutamine synthetase (GS) and glutamine:2-oxoglutarate aminotransferase (GOGAT) isoforms as are employed in primary ammonia assimilation (Hirel & Lea, 2002). Photorespiration also involves reductant cycling: NADH is produced by Gly oxidation in the mitochondria and it is required in equal amounts in peroxisomal glycerate synthesis.

The ability of chloroplasts or mitochondria to deliver NAD(P)H to the cytosol could be crucial in determining the rate of N assimilation (Krömer & Heldt, 1991; Hanning & Heldt, 1993). Low photosynthesis rates limit NR activity through decreased reductant availability, as well as through post-translational inactivation (Kaiser *et al.*, 2000; Foyer *et al.*, 2009). Nitrate reduction is significantly stimulated by anoxia and is favoured in conditions in which the complex redox cycling of photorespiration is active. Moreover, because amino acid synthesis requires simultaneous C oxidation and nitrate reduction, any perturbation of redox cycling could radically alter C–N partitioning through concerted and opposing effects on the two processes. Organic acid synthesis may also be subject to redox modulation in the C–N interaction because the production of compounds such as 2-OG requires oxidation through respiratory pathways involving the cytosol and mitochondria (Hodges 2002; Foyer *et al.*, 2003, 2006; Noctor *et al.*, 2007).

12.4.1 Up-regulation of the photorespiratory pathway in the CMSII mutant

The mitochondrial electron transport chain oxidizes NADH generated by various soluble mitochondrial enzymes, notably glycine decarboxylase and TCA cycle dehydrogenases (Rasmusson *et al.*, 2004; Noctor *et al.*, 2007). Mitochondria isolated from CMSII leaves have a decreased capacity for glycine oxidation (Sabar *et al.*, 2000), possibly due to increased redox control over glycine decarboxylase. Despite this, the glycine–serine ratio is not increased in leaves of the CMSII mutant in air (Dutilleul *et al.*, 2003, 2005), although a decreased mesophyll conductance suggests that the mutant shows higher rates of photorespiration than WT leaves.

There is a close relationship between primary N assimilation and photorespiration in C_3 plants (Stitt *et al.*, 2002). Four amino acids are directly involved in photorespiration: glycine and serine are involved in both the C and N cycles, while glutamate and glutamine participate in ammonia recycling (Keys *et al.*, 1978). The suppression of photorespiration by growth at high CO_2 reversed part of the CMSII metabolic signature, and resulted in decreases in glycine, serine and glycerate (Hager *et al.*, 2010). The metabolite signature of the CMSII leaves is thus due to an overall up-regulation of the photorespiratory C pathway, rather than other factors such as increased control at the level of glycine decarboxylase. CMSII-dependent changes in other amino acids and in compounds such as fumarate and shikimate were also independent of photorespiration.

12.5 Regulation of pyridine nucleotide metabolism in CMSII leaves

The total NAD plus NADH pool was greatly increased in CMSII plants relative to the WT (Dutilleul *et al.*, 2005). The size of the leaf NAD plus

NADH pool is influenced by metabolic (Shen *et al.*, 2006) and developmental triggers (Queval & Noctor, 2007) and also by environmental cues such as short-term differences in CO_2 supply (Igamberdiev & Gardeström, 2003). The total NAD(H) pool can vary more than the redox state (NADH–NAD^+ ratio) in conditions which influence plant growth and development such as the response to N availability (Hager *et al.*, 2010).

NAD is much more than a housekeeping redox carrier. For example, NAD cleavage is required for repair processes and in cellular signalling (Lin *et al.*, 2000; Berger *et al.*, 2004; Sánchez *et al.*, 2004; Vanderauwera *et al.*, 2007; Yang *et al.*, 2007). The pyridine moiety of NAD can be synthesized in plants from quinolinate or recycled from nicotinamide via nicotinate (Katoh *et al.*, 2006; Hunt *et al.*, 2007; Wang & Pichersky, 2007). While GC-MS does not readily measure nucleotides because they are relatively large ionic metabolites that are difficult to volatilize, the technique can detect simpler pyridine molecules. A strong correlation was observed between leaf NAD and leaf amino acid contents (Hager *et al.*, 2010), which could suggest an underlying cause–effect relationship. For example, a correlation between leaf NAD and leaf amino acid contents could indicate co-regulation of pyridine nucleotide and organic N status. The NAD molecule contains seven N atoms within the pyridine and adenine moieties. Our current knowledge of pyridine and purine nucleotide synthesis suggests that the atoms are derived from aspartate (3 N), glutamine (3 N) and glycine (Noctor *et al.*, 2006; Zrenner *et al.*, 2006). Although NAD is relatively minor to the plant N economy, it is possible that mechanisms could have evolved that link the synthesis of this essential co-factor to the availability of organic nitrogen. In addition, NAD(H) may have a role in the regulation of N assimilation. This might explain why the CMSII mutation does not display enriched amino acid contents under conditions of N limitation (Hager *et al.*, 2010).

12.6 CMSII is an N-sensing/signalling mutant

The modified respiratory pathways in the CMSII mutant compared to those of the WT leaves (Sabar *et al.*, 2000) result in enhanced rates of primary N assimilation and enrichment in amino acids under conditions of optimal N nutrition (Dutilleul *et al.*, 2005). Nitrate, amino acids and other N-containing metabolites play important roles in the regulation of plant architecture and resource allocation. It has long been recognized that nitrate is not only a substrate for N assimilation and an important component in the N-dependent regulation of gene expression (Palenchar *et al.*, 2004) but also as a potent signal that affects organ growth and development and the partitioning of biomass between roots and shoots (Scheible *et al.*, 1997; Forde, 2002; Foyer *et al.*, 2003). N also influences the extent of root branching (Scheible *et al.*, 1997), notably through its effects on lateral root (LR) formation (Forde, 2002). The analysis of NR mutants revealed that the shoot nitrate is an important signal regulating

shoot–root N allocation (Scheible *et al.*, 1997). Moreover, N signals interact intimately with C signals, particularly sucrose, glucose and trehalose, as well as organic acids such as 2-oxoglutarate (Finkelstein & Lynch, 2000; Finkelstein & Gibson, 2002; Leon & Sheen, 2003; Palenchar *et al.*, 2004).

LR formation in WT *N. sylvestris* seedlings shows similar responses to C and N availability as those described previously in *Arabidopsis* (Zhang *et al.*, 1999; Zhang & Forde; 2000, Signora *et al.*, 2001). In marked contrast to the WT, the CMSII plants produced no LRs during the first 7 days of seedling establishment regardless of the N or sugar source available (Pellny *et al.*, 2008). Moreover, even though in the longer term CMSII plants produced just as many LRs as the WT, the presence of external nitrate did not promote LR formation in CMSII, except when seedlings were grown on high C, when a weak response was observed. Models of N signalling and LR formation developed in *Arabidopsis* suggest that nitrate is the most important external signal, with an additional level of regulation arising from internal/metabolic C–N status mediated through tissue amines and carbohydrates (Zhang & Forde, 2000).

High contents of amines or certain amino acids reflect N sufficiency and act to prevent excessive LR growth (Zhang & Forde, 2000). Unlike the CMSII shoots, where leaf amino acid levels vary with the level of N nutrition supplied during growth, the roots of the CMSII mutant always show enhanced amine contents, regardless of environmental N availability. Enhanced root amine contents correlate with the poor LR growth in CMSII seedlings and the absence of stimulation of LR growth in the presence of external KNO_3, except at high sucrose (Pellny *et al.*, 2008). This finding suggests that the high internal N–C status of the mutant represses LR formation. On this basis CMSII could have been identified as a nitrate-sensing mutant in classical selection screens for C–N reciprocal sensing (Pellny *et al.*, 2008).

To investigate whether N-dependent regulation of the partitioning of biomass between roots and shoots was also altered in the CMSII mutants, plants were grown for 10 weeks on vermiculite with either optimal (5 mM NH_4NO_3; HN) or limiting (0.1 mM NH_4NO_3; LN; Pellny *et al.*, 2008). Under HN, CMSII plants were smaller and accumulated less biomass than the WT (Fig. 12.3; Pellny *et al.*, 2008). After 10-week growth under the HN conditions, the CMSII shoots and roots were about 3.5-fold smaller than those of the WT, as determined by both fresh weight and dry weight (Pellny *et al.*, 2008). In contrast, following growth at LN, where root biomass was also smaller in the CMSII plants than in the WT (~2.5-fold less), there were negligible differences in shoot biomass between the two genotypes (Pellny *et al.*, 2008). Hence, while growth under LN conditions significantly decreased the shoot–root ratio in WT plants, CMSII plants continued to invest even less (rather than more) biomass in the roots (compared to shoots) under conditions of N deficiency. Shoot C–N ratios were increased 2.5- to 3-fold under the LN conditions in both genotypes (Pellny *et al.*, 2008). However, the root C–N was lower in the mutant than in the WT under both growth conditions. Indeed, the CMSII

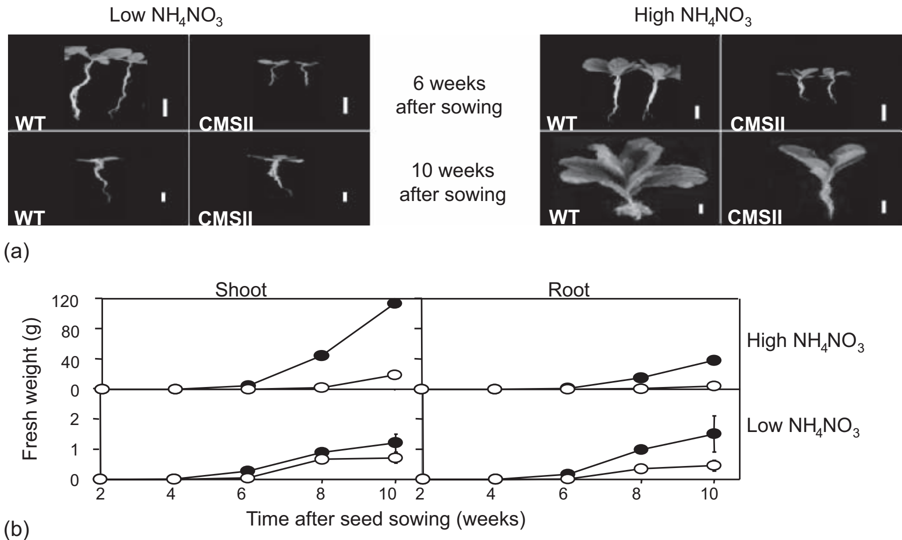

Figure 12.3 (a) The phenotypes of WT and CMSII *N. sylvestris* plants grown for 10 weeks at different levels of N nutrition (either 0.1 mM NH_4NO_3 or 5 mM NH_4NO_3). (b) Total shoot and root biomass (fresh weight per plant) at 10 weeks. Black circles, shoot; white circles, root.

root C–N ratios at LN were not significantly different than those of the WT roots at the HN growth condition. The lower root C–N ratios in CMSII are consistent with the view that this mutant is N-rich, based on profiling of leaf C and N metabolites (Dutilleul *et al.*, 2005). Both nitrate and total free amines were enriched in CMSII shoots and roots compared to WT when plants were grown at HN, but under the LN conditions, the only enriched soluble N pool in CMSII was the root amine pool (Pellny *et al.*, 2008). These data strongly suggest that the root amines–nitrate ratio has a strong influence on shoot–root ratios.

12.7 Regulation of gibberellin metabolism and signalling in the CMSII mutant

In addition to the effects on plant C–N relationships and signalling (Dutilleul *et al.*, 2005; Pellny *et al.*, 2008) discussed above, Complex I deficiency in the CMSII mutant results in a conditional slow-growth extended lifespan phenotype (Dutilleul *et al.*, 2003, 2005; Pellny *et al.*, 2008). However, the WT growth phenotype of the shoot was partially restored in Complex I-deficient seedlings by treatment with bioactive gibberellins (Fig. 12.4; Pellny *et al.*, 2008). Gibberellins (GAs) are essential endogenous regulators of plant growth and development that stimulate elongation or expansion of organs via enhancement of cell elongation and, in some cases, also cell division. GA synthesis and

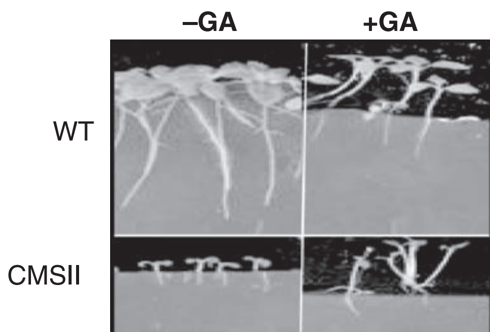

Figure 12.4 The response of WT and CMSII seedlings to bioactive gibberellins (GA). Seeds were germinated on medium supplemented with 1 mM NH_4NO_3 in the absence or presence of 10 μM GA.

signalling respond to environmental cues, including changes in light conditions, temperature or stress. For example, phosphate limitation-dependent changes in root architecture, particularly increased root hair growth, are at least partly the result of a reduction in the levels of bioactive GAs (Jiang *et al.*, 2007). GA metabolism is influenced by N availability and also by the alterations in NAD(H) availability and cellular metabolism/redox state caused by the absence of Complex I in the CMSII mutant (Pellny *et al.*, 2008). For example, the abundance of transcripts encoding the GA-inactivating gene *GA2ox* and the biosynthetic gene *GA3ox* was greatly enhanced under N deficiency compared to N-replete conditions. However, the abundance of *GA20ox* transcripts was largely unaffected by N abundance (Pellny *et al.*, 2008). A survey of nitrate-induced changes in gene expression in *Arabidopsis* using Affymetrix microarrays (Scheible *et al.*, 2004) revealed that *AtGA2ox2* transcripts were decreased within hours of nitrate treatment. Moreover, *AtGA3ox1* transcripts were more abundant after nitrate treatment, while there was little change in expression of the other GA metabolism gene.

Growth at LN resulted in reduced levels of several GAs, including bioactive GA_1 (Pellny *et al.*, 2008). Complex I deficiency in the CMSII mutant also caused large changes in the levels of the GA-biosynthetic intermediates GA_{19} and GA_{20} under HN conditions although the concentration of the bioactive GA_1 was not greatly affected, perhaps as a result of the homeostatic mechanism producing, for example, increased expression of *GA3ox* (Pellny *et al.*, 2008). These data provide evidence for cross-talk between N and redox signals in the control of GA synthesis and metabolism.

As discussed above, the CMSII mutation is associated with increases in leaf NAD(H) pools and amino acid contents when plants are grown under optimal nitrogen (HN) nutrition (Dutilleul *et al.*, 2005; Pellny *et al.*, 2008). However, the CMSII-dependent increase in leaf amino acid contents was absent under LN conditions (Pellny *et al.*, 2008; Hager *et al.*, 2010). Similarly, the NAD(H) pools were not increased in CMSII relative to the WT when plants were grown

at LN (Hager *et al.*, 2010). Under the LN conditions, where the pyridine nucleotide and amino acid pools are similar in the leaves of both genotypes, bioactive GAs are more abundant in CMSII leaves (Pellny *et al.*, 2008). This observation suggests that the root amine level influences GA metabolism in the shoots, enhancing both GA synthesis and turnover, presumably in an attempt to drive shoot growth. It is worthy of note that the stimulatory effect on bioactive GA accumulation is decreased at HN, where leaf NAD(H) and amino acid contents are high in CMSII leaves compared to the WT (authors' unpublished data). The enhanced GA metabolism in CMSII leaves is entirely consistent with the altered expression of antioxidant enzymes, diminished levels of reactive oxygen species and enhanced tolerance to both biotic and abiotic stresses in the mutant compared to the WT (Dutilleul *et al.*, 2003; Achard *et al.*, 2006).

12.8 Concluding remarks

Nitrogen is a major factor limiting plant growth in the field. At present, crop productivity is dependent on high nitrogen fertilization rates, but this practice is costly and has negative environmental impacts. An enhanced understanding of the mechanisms that determine nitrogen use efficiency (NUE) is required, together with the identification of new targets for plant improvement. Factors associated with C and N metabolism have a close relationship to yield, and they are thus important current targets for such analyses, particularly using quantitative trait loci. In addition, forward and reverse genetic approaches coupled to classical biochemistry and physiology are essential in advancing our current understanding of the enormous complexity of the C–N interaction and its intimate association with the plant signalling pathways that regulate growth and development. The characterization of the *N. sylvestris* CMSII mutant, which has an N-rich phenotype with altered 'nitrate-sensing' abilities and which shows changed responses in biomass partitioning to nitrate availability, reveals several new potential targets for improved NUE. The first potential target is the partitioning of NADH utilization between Complex I and the alternative respiratory dehydrogenases, which has a strong influence on plant N assimilation and amino acid accumulation. The second potential target is GA synthesis and signalling, which are modified both by N availability and by NADH metabolism within the plant cells. The third potential target is the root amine–nitrate ratio, which influences the partitioning of biomass between shoots and roots and the pathways of GA metabolism and signalling.

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Chapter 13

THE UTILIZATION OF NITROGEN BY PLANTS: A WHOLE PLANT PERSPECTIVE

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Abstract: The growth of whole plants is dependent on tissue N concentration. Plants that grow in N-rich environments have higher internal N concentrations and a higher relative growth rate, as described by the N productivity concept. When supply of nitrate N is interrupted, growth is maintained from storage pools of unassimilated nitrate for a limited time. Plants with low N supply show low shoot growth, high root–shoot ratio and decreased leaf growth. N is allocated more to the uppermost leaves, where there is a higher requirement for photosynthetic enzymes and chlorophyll, and mobilized to seeds as the leaves senesce. In actively photosynthesizing leaves, N-containing primary metabolites are present in relatively constant concentrations, although concentrations of N-containing secondary metabolites are more variable. The acquisition of nitrate is largely dependent on mass flow across the soil, but acquisition of ammonium depends more on diffusion to the roots. In a crop, uptake over a growing season is regulated by plant demand. This demand is signalled from the shoots to the roots. Some adaptations of roots enable the acquisition of nitrogen before it is acquired by other plants, and species adapted to N-rich soils have high leaf N concentrations and high specific leaf area, and out-compete other species for light. Those plants with higher leaf N concentrations seem to be more susceptible to herbivory and attack by micro-organisms, however.

Keywords: N cycling; N productivity concept; photosynthesis; resource availability hypothesis; root–shoot ratio; specific leaf area; storage pool

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13.1 Introduction

Our modern understanding of the importance of nitrogen to plants started in 1804 with the work of de Saussure, who showed that nitrogen is taken up by plant roots. In pioneering work, growing plants in water culture, he found that nitrates, amongst other salts, were essential for plant growth (Hewitt, 1966). Prior to that time it was thought that plants took up N in water-soluble organic compounds arising from humus, but de Saussure's work, and subsequent work by Sprengel, showed that soluble inorganic salts are the sources of plant nutrients, including nitrogen (van der Ploeg *et al.*, 1999).

We now know, in no small part due to Sprengel, that plants mainly acquire their N as the nitrate (NO_3^-) or ammonium (NH_4^+) ion. These are taken up predominantly through the roots, although in some instances ammonium ions arising from atmospheric pollutants dissolved in surface moisture on plants may enter through the foliage. In addition, a small number of plants (principally in the family Leguminosae/Fabaceae) are able to utilize dinitrogen (N_2) gas. However, in this instance they actually take up ammonium arising from the N_2 through the actions of symbiotic *Rhizobium* bacteria in root nodules. There is also some uptake of amino acids by plants. Different plants take up nitrate and ammonium to different extents, although nitrate is the predominant nitrogen form in agricultural and other fertile soils, and most plants preferentially take up this ion. A few plants, particularly those adapted to acid or waterlogged soils where ammonium is the predominant N form, take up ammonium preferentially, but for other plants, uptake of large amounts of ammonium can be harmful (Kronzucker *et al.*, 2001). However, for those plants that take up nitrate preferentially, the uptake of a small amount of ammonium with the nitrate gives them better growth (Cox & Reisenauer, 1973).

It was recognized before the modern era that the rate of nitrogen supply influences plant growth. In the 1660s and 1670s, it was known that plants benefit from potassium nitrate, and that the fertility of the land in respect to potassium nitrate affected yields of sugar (Barker, 2007). To this day, nitrogen remains the element applied to crops as fertilizer in the largest amounts, with NPK compound fertilizers being the most commonly used fertilizers worldwide and N being the major component of these.

13.2 Nitrogen and plant growth

Within a plant, N is the most abundant element after H, C and O. The C–N bond is stable, although not so stable that it cannot be broken in biochemical rearrangements, and many plant constituents contain nitrogen. The nitrate and ammonium taken up by plants are converted into the amino N component of amino acids, which are themselves the building blocks of plant

Table 13.1 Yield of fresh tubers of potato (*Solanum tuberosum*) at six rates of nitrogen supply at three harvest dates in two seasons (t/ha)

N application (kg N/ha)	1983			1984		
	68 days	**80 days**	**102 days**	**79 days**	**91 days**	**112 days**
0	23.3	22.8	31.7	30.2	31.2	36.9
50	29.4	31.0	35.8	40.3	37.4	46.8
100	28.7	30.8	44.0	40.9	44.6	51.6
150	27.6	31.5	45.7	41.5	43.2	56.3
200	33.3	30.3	45.5	38.3	42.0	54.7
250	30.3	32.4	43.6	39.2	40.7	54.7
S.E.	1.60	1.38	1.55	1.36	2.32	1.47

Reproduced from Millard and Marshall (1986), with permission of Cambridge University Press.

proteins. N is additionally found in nucleic acids, coenzymes, various secondary metabolites and chlorophyll.

Because of its pre-eminent role in so many major metabolites, the more nitrogen that a plant takes up, the more the plant grows. This is known from many classic studies. For example, application of nitrogen (as 'nitro-chalk') to potato (*Solanum tuberosum* L.) crops in two seasons at rates of up to 250 kg/ha gave noticeably increased yields of tubers in both seasons (Table 13.1; Millard & Marshall, 1986). The response to nitrogen supply shown here largely follows the law of diminishing returns, with supply of an increment of extra nitrogen at an already high rate of N application giving a smaller increment of extra crop yield than supply of an increment of nitrogen at low rate of application, and very high rates of N application giving no further yield increases. This is the standard response of crop yield to supply of nitrogen, and forms the basis of the Mitscherlich curves used in recommendations for the rate of N fertilizer application.

The uptake of nitrogen, either as nitrate or ammonium, requires energy, which is derived from photosynthesis. The nitrate taken up is reduced to nitrite and then to ammonium in enzyme-catalysed reactions that require energy, and the ammonium is added to organic acids (themselves products of reactions that follow from the Calvin cycle of photosynthesis) to make amino acids. The amino acids are assembled into proteins, which are used as enzymes for metabolism or as part of the structures of the plant. Ammonium taken up from the rooting medium is assimilated into amino acids in the roots (Findenegg *et al.*, 1989), whereas nitrate is mostly assimilated in the shoots, with a small proportion being assimilated in the roots, although the proportions assimilated in the two plant parts vary from species to species (Pate, 1973). The proportion of nitrate reduced in the roots is higher if plants are subjected to nitrate deficiency (Sutherland *et al.*, 1985). Furthermore, following resupply of nitrate to N-deficient plants assimilation occurs preferentially in the roots (Lancien *et al.*, 1999).

During the vegetative phase of growth the roots and shoots of plants are, in effect, physiological sinks for nitrogen (Hirel *et al*., 2007). Young leaves are sinks for both carbon and nitrogen, the former coming from mature leaves and the latter coming ultimately from the roots. Once mature, they are physiological sources of fixed carbon. In later stages of plant growth, usually after the onset of flowering, remobilization of N takes place as proteins are hydrolysed and the leaves (and to a certain extent the roots) become strong physiological sources of amino acids (Hirel *et al*., 2007). The developing storage organs are the sinks at this time. N continues to be absorbed through the young roots as the storage organs develop. N taken up during vegetative growth of maize (*Zea mays* L.) is partitioned to roots, leaves and in particular to the stalk, but N taken up during later stages of growth is more directly partitioned to the ear (Ta & Wieland, 1992). In perennial plants, such as deciduous trees, the N in chlorophyll and proteins may be remobilized for use in other parts of the plant, possibly in the following season. In the aspen (*Populus tremula* L.), it has been calculated that up to 80% of the leaf nitrogen is mobilized out of the leaf before abscission (Keskitalo *et al*., 2005).

In plants in the vegetative phase of growth, and under steady-state conditions, growth is dependent on internal N concentration. This is expressed most clearly in the N productivity concept (Ingestad, 1982; Ågren, 1985; Ingestad & Ågren, 1992).

This concept was developed from use of the relative addition technique, in which plants were supplied nutrients at rates to match plant demand (Ingestad, 1979; Ingestad & Lund, 1979). If plants are grown in otherwise constant conditions, but with nitrogen supplied at a rate that increases exponentially, the mass of the plants increases exponentially (i.e. the relative addition of nitrogen and relative growth rate are constant). By varying the extent of the daily increase (different relative addition), the plants are forced into growth patterns that show ever-increasing growth rates, but rates that still fit natural patterns and give constant relative growth rates (RGRs). In this way, plant growth, although constrained by the rate of N supply, is otherwise normal. Plant metabolism in respect to nitrogen appears to be in a steady state, and there is a linear relationship between plant N concentration and RGR, with the slope of the line representing this relationship being nitrogen productivity (P_n).

$$\mathrm{d}W/\mathrm{d}t \times 1/W = P_n \times (C - C_{min})$$

where W indicates dry mass; t, time; C, internal N concentration; and C_{min}, minimum N concentration below which it has no effect on plant growth.

If plant growth is entirely dependent on the rate of N supply, then the implication is that normally it is only nitrogen supply that limits plant growth. However, that cannot be an entirely accurate summary of the situation. If there were unlimited supply of water, energy and other nutrients, then this would be true, but that is rarely the case. With C, H, O and N being the

major elemental components of plant tissues, there is a balance between carbon metabolism and nitrogen nutrition, but increase in the ability of a plant to assimilate carbon usually leads to an increase in its ability to acquire N, and increase in N supply usually results in an increase in its ability to assimilate carbon. Under the circumstances of nitrogen being supplied by the relative addition technique, the ever-increasing daily supply gives the plant the opportunity to generate additional leaf material for capturing and assimilating carbon, so the balance between carbon and nitrogen metabolism is maintained over long periods.

What happens to plant growth under conditions where these steady-state conditions are disturbed, such as would occur with sudden loss of N supply, is seen in the nitrogen interruption experiments of Burns and co-workers (Burns, 1994a, 1994b; Burns *et al.*, 1997; Walker *et al.*, 2001). In these experiments, plants removed from nitrogen supply continued to grow, but at a slower rate. If the RGR is plotted against internal N concentration, the response to removal of nitrogen is curvilinear, and displays two phases. All the time that total N concentration in the plants remained high, RGR only declined slightly, but eventually the ongoing growth led to dilution of N concentration in the plants, and there reached a concentration of internal N below which RGR declined sharply (Fig. 13.1).

The reason that the plants in Burns' experiments kept growing immediately after removal from N supply is because they contained pools of N that could be mobilized for reuse in the plants. Could these be pools of stored nitrate still to be assimilated? Under some circumstances 'luxury consumption' of nitrate occurs, and plants take up nitrate at a faster rate than it can be assimilated. The excess nitrate is sequestered in storage pools (Ferrari *et al.*, 1973) that seemingly buffer the plant against periods of poor N supply (Bellaloui & Pilbeam, 1991; Richard-Molard *et al.*, 2008). These pools are most likely in the vacuoles of the leaf cells (Granstedt & Huffaker, 1982), with access of the nitrate to them being through channels in the tonoplast (De Angeli *et al.*, 2006). However, in the nutrient interruption model of Burns, the rate of reduction of stored nitrate did not maintain enough assimilated nitrogen for new growth at the rate occurring before the nitrogen was withdrawn, even when the nitrate concentration in the plants was high (Burns, 1994b), so these storage pools could not completely buffer plants against loss of N supply. In fact, nitrate concentrations dropped very rapidly, within 5 days of withdrawal of nitrate supply from lettuce (*Lactuca sativa* L.), and organic nitrogen concentrations declined over approximately 10 days, as dilution due to the ongoing growth of the plants occurred (Walker *et al.*, 2001; Fig. 13.1). In a study of glasshouse-grown hydroponic tomato (*Solanum lycopersicum* L. = *Lycopersicon esculentum* Mill.) plants in which nitrate constituted approximately 20% of the nitrogen in the vegetative tissues, withdrawal of nitrate from the nutrient solution led to a depletion of tissue nitrate to zero over about 45 days, indicating a role of this nitrate as a storage pool (Le Bot, *et al.* 2001). However, depressed growth of the plants was seen after about 2 weeks, so this slow rate of withdrawal

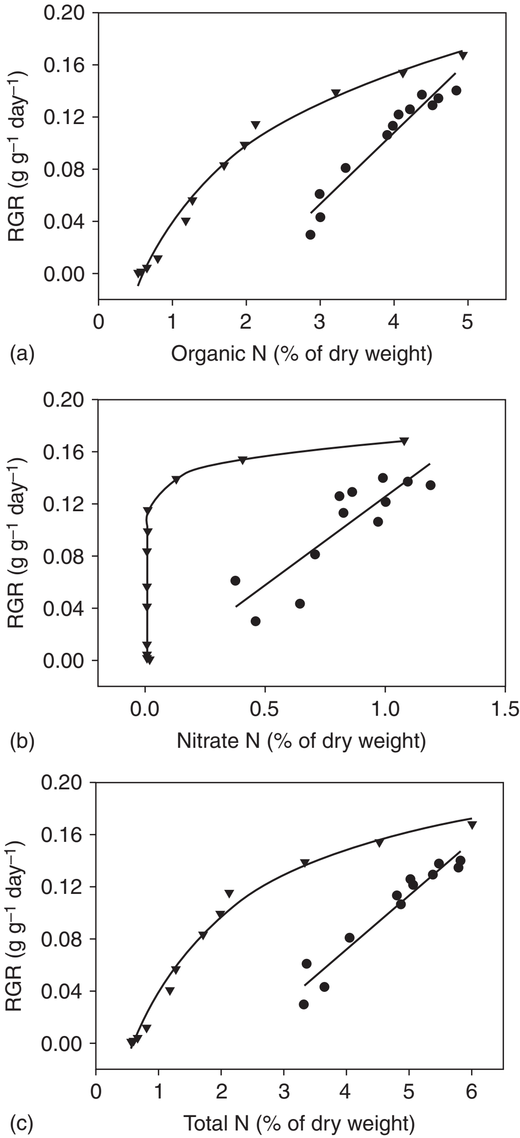

Figure 13.1 Relative growth rates of lettuce (*Lactuca sativa*) plants grown with continual supply of nitrate (•) or after nitrate was removed from the nutrient solution (▼), in relation to organic nitrogen concentration in the tissues (a), tissue nitrate concentration (b) and total N concentration in the tissues (c). (Reproduced from Walker *et al.*, 2001, with permission of Oxford University Press and the Society for Experimental Biology.)

did not enable the plants to withstand a long period of N deficiency without adverse effect. The sizes of these pools of unassimilated nitrate change over shorter periods as nitrate assimilation is faster than the rate of nitrate uptake during the early part of the light period, but during the night the rate of uptake is faster than assimilation, which virtually ceases (Matt *et al*., 2001). This has the effect that the storage pools of nitrate are replenished during the dark, and become depleted during the light (Ter Steege *et al*., 1999; Matt *et al*., 2001). According to the nitrogen productivity concept, the whole idea of nitrate occurring in 'storage pools' can be regarded as being redundant, as a plant can redistribute its resources to optimize growth, and there is no partitioning between metabolic and storage pools (Ingestad & Ågren, 1992).

Regardless of whether or not plant nitrate is seen as being a store of N for future use by the plant, these pools of unassimilated nitrate still have an important role in leaf expansion and maintenance of turgidity, because of their osmotic potential. In the experiments of Walker *et al*. (2001), leaf water contents dropped sharply with the decline of shoot nitrate concentration. In spinach (*Spinacia oleracea* L.) leaves, expansion can occur during the night due to increased nitrate concentrations, substituting for soluble carbohydrates and maintaining osmotic potential to such an extent that turgor remains high (Steingröver *et al*., 1986). In a field experiment on potato, nitrate in the lower leaves at early stages of growth accounted for up to 35% of the total osmotic potential of young potato leaves (Millard & Catt, 1988). In monocotyledonous tall fescue (*Festuca arundinacea* Schreb.), there was shown to be low nitrate concentration in the zone of the leaf closest to the stem, the site of the intercalary meristem where cell division occurs, but it was at highest concentration in the area of rapid cell expansion further away from the stem (Gastal & Nelson, 1994). This accumulation was partly a consequence of the fact that the activity of nitrate reductase, the first enzyme in the sequence of reactions in the assimilation of nitrate, was highest further along the leaf blade. This has consequences for the provision of leaf area in relation to N supply (see Section 13.3.2).

There is some debate about the relevance of the Ågren/Ingestad and Burns' approaches to investigating the relationship between N supply and plant growth. The relative addition technique is rather artificial in that plants are forced into a pattern of growth by the rate of nitrogen application. It can be argued that plants in a non-experimental environment would not have their growth constrained in this manner, and might grow differently. However, the N interruption experiments are also artificial as in a non-experimental environment plants are unlikely to be suddenly subjected to total withdrawal of nitrogen. Plants are able to adapt to their environment to maximize resource acquisition, including their acquisition of nitrogen (see Section 13.5), so sudden N deficiencies are unlikely to occur naturally, or even in an agricultural environment. We have become conditioned to a situation in which experimental plants grown in a nutrient solution, or horticultural crops in

hydroponics, can be suddenly deprived of nitrogen. More normally, plants develop to continue acquiring N, partly by changes in partitioning of biomass and in their use of N-containing metabolites, so in this respect plants grow more like those in the relative addition experiments over long periods. In the experiments of Walker *et al*. (2001), the control plants (those that were not subjected to nitrogen interruption but continued to receive nitrogen at rates above their requirements) showed a linear relationship between internal N concentration and RGR (Fig. 13.1), and these may be more like plants growing in environments where they have the ability to acquire nitrogen through changes in partitioning and physiology.

13.3 Nitrogen, biomass partitioning and yield

13.3.1 Shoot–root partitioning

When plants grow under steady-state conditions regarding N supply, as in the relative addition experiments, their overall proportions remain approximately constant. As developing seedlings age, there is an initial increase in proportion of root systems, but after this plants with an adequate supply of resources maintain roughly constant proportions throughout vegetative growth, although with a slowly declining proportion of roots (Wilson, 1988), until there is an increase in reproductive structures after the onset of generative growth.

However, if during the vegetative phase plants experience a shortfall in N supply, not only does their overall growth rate fall but the root fraction increases. This is because shoot growth is decreased more than root growth by shortage of N (Brouwer, 1962; Ericsson, 1995). As a consequence, plants subjected to low N maintain root growth but suffer a decrease in shoot growth. The size of the shoot fraction is directly related to internal N concentration (Ågren & Ingestad, 1987; Levin *et al*., 1989; Ågren & Franklin, 2003), so root fraction must obviously also be directly affected by internal N concentration, but in the opposite way. The situation is somewhat different for plants with storage roots, and in the biennial plant species *Cirsium vulgare* (Savi) Ten. it was shown that there was no difference in the proportion of the plant made up of the tap root plus the storage compounds (deemed to be total available carbohydrates, amino acids and the part of the soluble proteins that increased in concentration when storage was occurring) with different rates of nitrogen supply (Heilmeier *et al*., 1994). The proportion of fine roots, deemed to be those actively engaged in acquisition of resources, was higher in plants with low N supply.

If the roots have a primary role in acquisition of nutrients and water, the shoots have a primary role in interception of light energy and assimilation of carbon. Leaf area is important to the overall growth of plants as it has a

large impact on the amount of incoming radiation that plants can intercept. The yield of C_3 crops in northern Europe is strongly related to the amount of radiation intercepted, with slight increases in interception giving big increases in yield for plants that are intercepting light poorly, although only smaller increments of extra yield for plants that are intercepting light more efficiently (Greenwood *et al.*, 1986). It also seems to be the case that where shortage of nitrogen gives lower leaf growth it decreases stomatal conductance, which has a negative effect on the rate of carbon assimilation per unit leaf area (Broadley *et al.*, 2001).

If the bigger leaf area resulting from a greater N supply gives more interception of light, more photosynthesis and hence more biomass production, then it can be seen that plants optimize their growth by maintaining as large a shoot as possible. However, they have to provide a large enough root system to acquire the water, nitrogen and other nutrients required from the soil. There is, in effect, a 'functional equilibrium' (Brouwer, 1962; Davidson, 1969) between shoot and root growth to optimize a plant's acquisition of resources obtained from the atmosphere and resources obtained from the soil. This can be expressed by root mass × rate of nutrient absorption being proportional to leaf mass × rate of photosynthesis (Davidson, 1969).

The plant is able to alter the equilibrium between root and shoot activity by changes to one or more of the four parameters – root mass, shoot mass, root-specific activity (root activity per unit mass) and shoot-specific activity (shoot activity per unit mass). Changes to shoot mass would automatically change root mass in the opposite direction, so change in only one of the parameters has a big effect on the equilibrium.

During periods of N depletion there is a gradual decrease in the size of the leaf metabolic N pool (Caloin & Yu, 1984), and as there is a large amount of movement of nitrogenous compounds from shoots to roots in the phloem, and back to the shoots in the xylem (Simpson *et al.*, 1982; Jeschke *et al.*, 1985; Cooper & Clarkson, 1989; Larsson *et al.*, 1991), the degraded leaf N can add to this pool of N cycling within the plant for subsequent withdrawal into the roots. A large proportion of the nitrogen that fuels root growth under these circumstances comes from the degradation of ribulose bisphosphate carboxylase/oxygenase in the leaves (Lawlor *et al.*, 1989), and also from the enzymes of nitrogen assimilation in the leaves. Withdrawal of nitrogen from tobacco (*Nicotiana tabacum* L.) over 8 days resulted in the loss of ribulose bisphosphate carboxylase/oxygenase protein and activity of the enzyme from the leaves and a decline in the rate of photosynthesis (Paul & Driscoll, 1997). A further extent to which 'recycling' of nitrogen occurs in plants comes from the fact that NH_4^+ released from the formation of one molecule of serine from two molecules of glycine during photorespiration is reassimilated by leaf cytosolic glutamine synthetase (Hirel *et al.*, 2007). This release of NH_4^+, and its reassimilation, actually seems to represent a greater N flux than the primary assimilation of nitrogen that is occurring at the same time (Tcherkez & Hodges, 2008).

It can therefore be seen that if plants subjected to low N maintain root growth but suffer a decrease in shoot growth, the driving force for this comes from changes in internal N concentration (Ågren & Ingestad, 1987; Levin *et al.*, 1989; Ågren & Franklin, 2003). The high root–shoot ratio of plants grown with low nitrate supply can be clearly explained by shoot growth being lower under low N conditions, thus making carbon skeletons and reduced nitrogen compounds available to maintain root growth. Earlier interpretation of the reason behind this phenomenon was that assimilation of nitrate in the roots made fixed N available to the root system before 'excess' fixed N was exported to the shoots, but this interpretation did not account for the fact that the shoots might therefore be expected to have first call on the assimilated carbon compounds. In experiments on nitrate reductase-deficient pea (*Pisum sativum* L.), in which roots or shoots of the mutants were grafted on to wild-type plants to give plants that had nitrate reductase activity localized in either roots or shoots, or in both plant parts, under low N supply root–shoot ratio of the plants was higher than under high N supply, but the ratio was similar irrespective of where the nitrate reductase activity was located (Lexa & Cheeseman, 1997). This clearly demonstrates that the site of the assimilation of nitrate has no effect on root–shoot ratio. The assimilation of nitrate in the roots probably has more effect on very localized root growth as when there is unequal distribution of nitrate to a root system, although the overall size of the root system that results is the same as with more even supply, there is different dry matter allocation within the root system itself (Öhlén & Larsson, 1992).

What actually determines the proportion of shoot and root at the cellular level? We can envisage a situation where shoot growth is controlled (by regulation of the activity of meristems in the shoot) and root growth uses the remaining resources. *N. tabacum* plants were transformed to give different amount of expression of nitrate reductase, and plants with very low nitrate reductase activity had much higher shoot–root dry weight ratios than wild–type plants with high nitrate supply (8–10:1 compared with 3.5:1) (Schieble *et al.*, 1997). High nitrate concentrations in the leaves inhibited starch synthesis and turnover, and gave decreased concentrations of sugars in the roots, and the leaf N content seemed to be a major factor influencing shoot–root ratio. In split-root experiments, root growth was inhibited by high nitrate concentration in the shoots in the halves, not supplied nitrate, but there was no effect from root nitrate concentration. In *Arabidopsis thaliana* (L.) Heynh., there was a strong correlation between nitrate and organic N and shoot growth, and between sucrose concentration and root growth (Schulze *et al.*, 1994). Control by nitrate concentration in the shoots would make sense based on the known role of nitrate as a physiological signal, but would not explain how the shoot–root ratio is determined in ammonium-fed plants (Andrews *et al.*, 2001; Andrews *et al.*, 2006), and the alternative suggestion is that leaf-soluble protein concentration is the determining factor influencing the amount of shoot growth (Andrews *et al.*, 1999, 2001, 2006). The relationship

between shoot–root ratio and leaf N concentration seems to be the same irrespective of if nitrate N or ammonium N is supplied for many species, but not all (Andrews *et al*., 2006). If shoot and root growth were limited by the availability of assimilated carbon and assimilated nitrogen, shoot growth would be enhanced by greater supply of nitrogen due to its proximity to the sites of carbon assimilation (Andrews *et al*., 2001, 2006). This implies that overall plant growth, irrespective of the partitioning into shoots and roots, is source-limited, which may not actually be the case (see later). How changes in shoot protein concentration would control shoot–root dry matter allocation at a cellular level is also not yet known. If we can discount nitrate as a signal, there is a possibility that changes in import of sucrose into the roots from the shoots might be a signal for root growth as well as a substrate (Hermans *et al*., 2006), and this might relate to the findings of Schieble *et al*. (1997) and Schulze *et al*. (1994). However, Andrews *et al*. (2007) point out that root growth is not correlated with leaf sucrose concentration across a range of rates of N supply, and the issue is a matter of current debate (Andrews *et al*., 2007; Hermans *et al*., 2007).

13.3.2 Leaf growth

Plants with poor N supply have small leaves, and fewer in number. Nitrogen-deficient plants also have a smaller leaf area duration due to premature senescence (Spiertz & de Vos, 1983), and in experiments on field-grown wheat (*Triticum aestivum* L.), either supplied N fertilizer or left without supplemental N, the flag leaves senesced a week earlier in the no-N treatment (Lawlor *et al*., 1989). A major consequence of this is that total plant photosynthetic capacity is lower than the potential capacity, and it is this lowered photosynthetic activity that gives rise to slower growth rate and smaller plants with shortage of N. Another consequence of less leaf growth is seen in the assimilation of N itself. The leaves are the major site of nitrate reduction in plants, with nitrate reductase activity being high in young leaves and then gradually declining as leaves age (Bellaloui & Pilbeam, 1990). An exponentially increasing leaf biomass during vegetative growth gives an exponentially increasing capacity to assimilate nitrate.

A lower leaf area with low N supply is very obvious in some species, and arises from there being much less leaf expansion (Muchow, 1988; Gastal *et al*., 1992; Gastal & Nelson, 1994). For example, tall fescue (*F. arundinacea*) was shown to have a leaf extension rate that increased with daily increase in temperature during the early part of the growing season, but at each temperature those plants supplied N at 200 kg/ha had a higher leaf extension rate than plants supplied N at 100 kg/ha, which in turn had higher leaf extension rate than plants supplied N at 50 kg/ha (Gastal *et al*., 1992). Similarly, potato has a slower rate of leaf expansion under N deficiency (Vos & van der Putten, 1998). In flag leaves of wheat, decreased leaf area with lower N supply was shown to be mainly due to lower cell number, and only to a small extent due

to lower cell volume (Lawlor *et al.*, 1989). In fact, the effect of N supply on leaf expansion is probably on both cell division and cell expansion. In lettuce it has been shown that lower cell division gives a smaller area of leaves that are formed after imposition of N deficiency, but leaves that were already formed before the imposition of deficiency ended up forming a smaller area due to cell expansion being reduced (Broadley *et al.*, 2001). Smaller cell size in mature leaves of *Ricinus communis* L. and *Helianthus annuus* L. gave small leaf areas when N deficiency was imposed, but lower rate of cell division gave lower leaf area in the younger leaves (Roggatz *et al.*, 1999; Trápani *et al.*, 1999).

In many cereals there is also an effect of nitrogen on tillering, with fewer tillers occurring with lower N supply, something that has a bigger effect on reducing total leaf area per plant than the smaller leaves (Spiertz & de Vos, 1983). This obviously also has the effect of decreasing leaf area index (LAI), and is the basis for the application of N fertilizer early in the season for winter cereals in northern Europe despite the potential loss of soluble nitrates from the soil during the autumn rains.

High shoot mass can give a high capacity to assimilate carbon, but this capacity is also a product of the assimilatory capacity per unit mass. Under conditions where N is not limited, the RGR is dependent on photosynthetic capacity, and high RGR can come from either high specific shoot activity and low shoot fraction or low specific shoot activity and high shoot fraction (Hilbert, 1990). There is a strong correlation between leaf N concentration and the photosynthetic capacity of individual leaves (Field & Mooney, 1983; Greenwood *et al.*, 1986). The relationship occurs because N is a key component of chlorophyll, the enzymes of the C_3 and C_4 assimilatory pathways, the electron acceptors and donors of the light reactions and the proteins in the membranes of the chloroplasts. The rate of carbon dioxide assimilation is strongly related to leaf N content, with chlorophyll content and activity of ribulose bisphosphate carboxylase being proportional to it (Evans, 1983). This is seen for many plant species, and for C_3 plants; although there are a range of values of both leaf N content and rate of carbon dioxide assimilation, the relationship between the two seems to be quite rigid (Field & Mooney, 1986). The enzyme ribulose bisphosphate carboxylase/oxygenase is the major protein of leaves, particularly in C_3 plants, because of its low catalytic rate (Lawlor, 2002), and it can account for up to 50% of the soluble protein in wheat leaves and 30% of the nitrogen (Lawlor *et al.*, 1989).

In experiments on field-grown wheat, plants with N fertilizer applied at 200 kg/ha had concentrations of soluble protein in their flag leaves of up to 14 g/m^2 at the time of maximum leaf expansion (of which nearly 7 g/m^2 was ribulose bisphosphate carboxylase/oxygenase protein) and chlorophyll concentrations of nearly 1 g/m^2 (Lawlor *et al.*, 1989). By contrast, in unfertilized plants soluble protein concentrations were approximately 5 g/m^2, ribulose bisphosphate carboxylase/oxygenase protein concentration was approximately 3 g/m^2 and chlorophyll concentration was nearly 0.4 g/m^2. The rate of photosynthesis per leaf area was higher with the higher concentrations

of soluble protein, ribulose bisphosphate carboxylase protein and chlorophyll (Lawlor *et al.*, 1989). A low rate of N supply to *A. thaliana* seedlings has been shown to result in lower levels of transcript for synthesis of chlorophyll *a*/*b*-binding protein and the small subunit of ribulose bisphosphate carboxylase (Martin *et al.*, 2002).

The proportions of N present in these different components of the light-dependent and light-independent reactions of photosynthesis vary according to the position of a leaf in the canopy and the level of irradiance it is exposed to. The interception of light down through the different layers of a canopy in a crop is described by the Monsi–Saeki equation (Hirose, 2005), which states that:

$$I = I_o e^{-KF}$$

where I shows photosynthetically active radiation intercepted on a horizontal plane; I_0, incident radiation at the top of the canopy; K, extinction coefficient; F, leaf area index (LAI). This effectively shows that there is an exponential decrease in radiation intercepted with increase in LAI (leaf area of plant or crop/land area under plant or crop), so that leaves at the bottom of a dense canopy (a canopy with a high LAI) receive only a small proportion of the radiation intercepted at the top of the canopy. Any investment of nitrogen into photosynthetic structures towards the bottom of a dense canopy would be less efficient than investing nitrogen at the top of the canopy, and it seems as if N is distributed through a canopy so that more of it is invested towards the top (Anten *et al.* 1995; Hirose, 2005):

$$n_{\mathrm{L}} \frac{K(N_{\mathrm{t}} - n_{\mathrm{b}} F_t) e^{-KF}}{1 - e^{-KF_t}} + n_{\mathrm{b}}$$

where n_{L} indicates leaf nitrogen per unit area; N_{t}, the total amount of leaf canopy nitrogen; n_{b}, leaf N per unit area that is not involved in photosynthesis; that is, photosynthetic N ($n_{\mathrm{L}} - n_{\mathrm{b}}$) decreases exponentially as a function of F where the proportionality constant is K, the light extinction coefficient. The n_{L} tends to be lower, the lower down a canopy you go. N is distributed to optimize photosynthesis in the whole canopy (Hirose, 2005), with there being lower concentrations down where light interception is less. This unequal distribution of N is shown by modelling to give bigger advantages to a plant in terms of enhanced total photosynthesis when the LAI is high (Schieving *et al.*, 1992) and where extinction coefficient is high (Anten *et al.*, 1995). In experiments to test the importance of unequal distribution of N where extinction coefficient is high, it was found that dicotyledonous plants (in this study, C_3 soybean, *Glycine max* (L.) Merr., and C_4 amaranth, *Amaranthus cruentus* L.), which have more horizontal leaves and hence a higher extinction coefficient than monocotyledonous plants (C_3 rice, *Oryza sativa* L., and C_4 sorghum *Sorghum bicolor* (L.) Moench), had more unequal distribution of nitrogen between their leaves (Anten *et al.*, 1995). In some monocotyledonous species, where the upright leaves may span the depth of the canopy, there

can be variable concentrations of N within individual leaves, depending on the height in the canopy of different regions of the leaf (Pons *et al.*, 1993).

Because a large proportion of plant N is used in the photosynthetic machinery, and photosynthesis functions essentially identically in all plants except for the distinction between C_3 and C_4 mechanisms, it is expected that there would be a similar relationship between the amount of 'photosynthetic' nitrogen and plant mass per unit area across a range of species. This should particularly be the case given that N is distributed in the canopy so as to optimize photosynthesis. In a study of C_3 crops in Europe, Greenwood *et al.* (1991) did indeed show a very similar relationship between the amount of N involved in photosynthesis and plant mass per unit area. There is a critical plant nitrogen concentration above which there are no further increases in crop yield (Greenwood *et al.*, 1986, 1990; Gastal & Lemaire, 2002), and this nitrogen concentration value is lower in big plants because there is an increase in the amount of structural carbohydrate and a decrease in the proportion of cytoplasm as plants grow. The nitrogen concentration value is typically lower in C_4 crops than in C_3 crops, probably because C_4 photosynthesis is more efficient than C_3 photosynthesis (Greenwood *et al.*, 1990).

Although plants differentially allocate their nitrogen to different levels within a canopy, this does not necessarily give rise to the differences in photosynthetic rate observed at the different levels. It is possible that it is the different rates of photosynthesis that occur at the different levels within the canopy that themselves determine how much nitrogen is allocated to each layer. In a study of Baltic tree and understorey species, photosynthesis was down-regulated in leaves adapted to sunny and shaded conditions (which contained different concentrations of N), indicating that the rate of photosynthesis was not dependent on N concentration but that N concentration could have been dependent on the rate of photosynthesis (Laisk *et al.*, 2005).

The overall control of N distribution is complex and requires further study at the molecular level. It is obviously intimately linked with carbon metabolism, and photosynthesis and nitrogen assimilation are interrelated. For example, expression of genes coding for some enzymes of C_4 photosynthesis is regulated by nitrogen availability (Yamazaki *et al.*, 1986). Activity of phosphoenolpyruvate carboxylase, which in addition to being the first enzyme of C_4 carbon assimilation feeds in carbon to the tricarboxylic acid (TCA) cycle in all plants and so allows organic acids to be removed for amino acid synthesis, is strongly regulated by nitrate through the action of phosphoenolpyruvate carboxylase protein kinase (Foyer *et al.*, 2003). Increasing N supply, particularly as nitrate, also stimulates phosphoenolpyruvate carboxylase gene expression (Pasqualini *et al.*, 2001). Expression of some Krebs cycle enzymes is affected by N supply (Lancien *et al.*, 1999).

As lower specific leaf nitrogen content (leaf N content per unit leaf area, SLN) results in there being less photosynthesis (Sinclair & Horie, 1989; Muchow & Sinclair, 1994; Anten *et al.*, 1995), it can be seen that the lowered plant productivity with N deficiency can be ascribed to lower shoot specific

activity. It certainly seems to be the case that in some species response to N deficiency is mostly seen as a decrease in leaf N content whereas in others the main effect is decrease in leaf area. In effect, there are two major responses to N deficiency here; either a decrease in leaf area, and hence a lowered ability to intercept radiation, or else the maintenance of leaf area, and hence the amount of radiation intercepted, but less ability to use this intercepted radiation productively (Lemaire *et al.*, 2008).

Maize is a species in which SLN is obviously lower (Muchow & Davis, 1988), and it has been suggested that in grasses leaf area remains high under N deficiency, and the N in the plants is present at lower concentration. In dicots, the opposite seems to occur, and low N supply results in a low leaf area but leaf N concentration (and photosynthetic capacity per unit leaf area) is maintained (Radin, 1983; Vos *et al.*, 2005). Indeed, in the potato plants where the positive effect of N supply on yield is highlighted in Table 13.1, there was little difference in radiation use efficiency (RUE, the total dry mass of the plants per unit of accumulated photosynthetically active radiation intercepted by them) between the different rates of N supply for the first 80 days of growth (Millard & Marshall, 1986). We can then envisage two strategies for plants to withstand shortage of nitrogen, one in which plants maintain leaf area but then have insufficient N to distribute in these leaves to maintain net assimilation rate (NAR), and one in which plants produce a smaller leaf area, but assimilation of carbon is maintained at a high rate within the leaves that are produced (Fig. 13.2). The examples already given show that both sorts of responses are seen in a range of plant species, and in Radin's experiments, NAR was noticeably decreased by lowered N supply in dicots as well as in monocots, and there was some reduction in leaf area with N deficiency in the monocots. It is probably not correct to think of plants having either lower LAI or lower radiation use efficiency in response to N deficiency, but that plants fall on a continuum between these responses. Oilseed rape (*Brassica napus* L.), for example, has both a decreased LAI and depressed RUE with N deficiency (Colnenne *et al.*, 2002). Similarly, despite the findings of Gastal *et al.* (1992), tall fescue has a lower RUE with lowered N supply as well as a lowered ability to intercept radiation (Bélanger *et al.*, 1992). However, in common with the general finding that monocots subjected to N deficiency protect leaf area rather than photosynthetic capacity per unit leaf area, RUE was lowered by shortage of N even when its supply was still relatively high, whereas the interception of photosynthetically active radiation was only substantially less when N supply was very low.

These differences in response to N deficiency were tested on datasets of production of maize, oilseed rape, sorghum, sunflower, tall fescue and wheat in Australia and France, and it was found that maize and tall fescue maintained their leaf area per unit of biomass, but took up less nitrogen per unit leaf area, under N-deficiency conditions, and oilseed rape and wheat had lower leaf area per unit of biomass but maintained the amount of nitrogen taken up per unit leaf area, and sorghum and sunflower were intermediate

Dicotyledon

High N

Low N

Monocotyledon

High N

Low N

Figure 13.2 Alternative strategies of change in leaf activity or leaf mass in relation to N deficiency. Suggestions of two approaches of plants to cope with shortage of nitrogen, from Radin (1983) and Vos *et al*. (2005). Size of leaves (open boxes) denotes leaf area, size of shaded circles denotes net assimilation rate.

Photons intercepted.

Photosynthates produced.

(Lemaire *et al*., 2008). In all the plant species, there was lower SLN in the low-N treatments than in the high-N treatments, indicating that the rate of photosynthesis per unit leaf area was likely to have been lowered by N deficiency. The extent to which the lowered leaf N concentrations contributed to lower plant yields must have differed between those species that maintained a high leaf area under N deficiency and those that did not.

In the oilseed rape and wheat plants, the SLN in the high-N treatments was approximately 3 g/m^2 in the early season, when LAI was low, and had decreased to approximately 1 g/m^2 around flowering, when LAI had increased to 6 in oilseed rape and almost 7 in wheat. Under N deficiency, although SLN was less, it never declined much below 1 g/m^2. The low values of SLN with high LAI could well be indicative that in dense canopies in these species there are more shaded leaves at the bottom of the canopy in which leaf N concentration is likely to be low. In maize, the value of SLN was relatively constant at 1.6–1.7 g/m^2 throughout the growing season in the high-N treatments, even when LAI had increased to as high as 4. Under N deficiency it dropped below 1 g/m^2. It seemed that maize required a critical value for leaf expansion, as first propounded by Muchow (1988), of 1.0 g/m^2 and that critical SLN for maximum uptake of nitrogen per unit LAI was 1.4–1.5 g/m^2, so N deficiency in maize does not prevent leaf growth (Lemaire *et al.*, 2008). It is a plant with a low leaf N content per unit leaf area, which allows it to use its nitrogen to produce a larger canopy, and it has a high radiation use efficiency when well nourished even with the low leaf N concentration (Sinclair & Horie, 1989).

13.3.3 Seeds and generative growth

If the amount of shoot growth is important for the rate of vegetative growth of a plant, and this is influenced by nitrogen affecting leaf growth and leaf duration, generative growth is dependent on the ability of the plant to remobilize its resources into developing seeds. In maize, those plants with the highest nitrogen use efficiency have a strong ability to remobilize fixed N in the leaves into the developing grains (Hirel *et al.*, 2001). Low N inputs give lower grain yield, not only due partly to individual grain weight being smaller, but also due to a smaller number of grains (Paponov *et al.*, 2005). This is caused by abortion of developing seeds, probably due to the shortage of photosynthates that results from the impaired N nutrition (Gallais & Hirel, 2004). Remobilization of N from the leaves to the developing grains involves the breakdown of ribulose bisphosphate carboxylase/oxygenase, and so occurs once senescence of a leaf has already commenced (Gallais & Hirel, 2004). For plants adequately supplied with N during the early stages of grain development, the continuing uptake of N is important in grain development, but with shortage of N (or commencement of leaf senescence for other reasons), uptake becomes increasingly less important. In maize, 45–65% of the N in grains comes from remobilization from the leaves, with the rest coming from uptake after silking (Hirel *et al.*, 2007). In fact, 60–85% of the N present in a plant at anthesis is located in the ear eventually (Ta & Wieland, 1992). The normal practice for nitrogen fertilization of maize is a single N application at sowing.

In experiments in which N shortage was imposed during the lag phase of kernel growth, a higher proportion of biomass was allocated to the roots

of maize, rather than the cobs, than in control plants (Paponov & Engels, 2005). Later, there was a higher amount of N that had been allocated to the grains than taken up after the N stress had been imposed, so this could indicate less development of grains due to the leaves not being a sufficient source of metabolites to maintain grain development. However, the soluble carbohydrate levels in the stems were higher than in N-replete plants, so it seems more likely that sink strength was lowered by N deficiency, and any effects on leaf area duration and lowered rates of photosynthesis were caused by feedback inhibition. The low N plants had high concentrations of sugars in the kernels during the lag phase of their development, again, indicating that their impaired development was not due to source limitation (Paponov & Engels, 2005). Furthermore, shading of a genotype that was not N-efficient, and supplied with low N from just after anthesis did not lower grain yield any further, again, indicating that the plants were not source-limited (Paponov *et al.*, 2005).

In wheat, 60–95% of grain N comes from remobilization, and uptake of N after anthesis is less important (Simpson *et al.*, 1983; Hirel *et al.*, 2007; Bancal *et al.*, 2008). High levels of N supply to wheat cause more initiation of spikelets, and as a consequence more grains per ear (Ewert & Honermeier, 1999). Late application of N also gives a bigger grain size (Forster, 1973), although it is unlikely that this is a primary effect of N supply rather than enhanced production of photosynthates. In the case of winter wheat and barley in northern Europe, late applications of N fertilizer in spring and early summer are used to prolong leaf area duration, which keeps a high nitrogen concentration in the flag leaf and maintains carbon dioxide assimilation in that leaf (Spiertz & de Vos, 1983). In flag leaves of wheat, the concentrations of total soluble protein and ribulose bisphosphate carboxylase/oxygenase protein fall from almost immediately after the leaves reach their maximum size, and drop to almost zero by senescence (Lawlor *et al.*, 1989), so later applications of nitrogen help maintain levels of this, and other, essential enzymes. Another effect of late application of nitrogen is that it increases grain protein concentration in wheat (e.g. Bancal *et al.*, 2008). The normal practice for N fertilization of winter wheat is for three applications – one early to promote tillering, one at the beginning of stem elongation and one at the second node stage (Hirel *et al.*, 2007) – although in many soils it is also common practice to supply N fertilizer to the seedbed. In rice, N from the vegetative organs accounts for 70–90% of the N in the panicle at harvest, and it is the N in leaf blades that had already accumulated before flowering which is used for grain filling (Hirel *et al.*, 2007). Like wheat, there is a positive effect of N supply on tillering, with higher N concentration in the culm (stem) giving rise to more tillers (Tanaka & Garcia, 1965). Rice typically receives N fertilizer (as ammonium or urea) at sowing, with an additional top dressing between formation of the panicle primordia and the late stage of spikelet initiation (Hirel *et al.*, 2007).

In the 'stay-green' phenotype of cereals, there is a higher leaf N content (they have thicker leaves, and hence lower specific leaf area (SLA) and higher SLN), there is a longer stability of some chloroplastic proteins and photosynthesis carries on in the leaves for longer before senescence (Borrell *et al.*, 2001). The leaves are more productive physiological sources, but there are also an increased number of grains per square metre, so the plant has also a stronger sink strength. As this sink demand is not matched by nitrogen being remobilized from the leaves, which are still actively photosynthesizing, the stay-green phenotype takes up more N from the soil for partitioning to the grains (Borrell *et al.*, 2001). It seems that the phenotype requires continual supply of nitrogen if the leaves are to remain green, but there is also some evidence that the stay-green phenotype may not necessarily be more efficient at uptake or remobilization of the nitrogen in the plant (Subedi & Ma, 2005). This contradicts the earlier work, but was based on a different stay-green cultivar.

It is an interesting point whether the formation of spikelets is due to increased source pressure (i.e. more export of photosynthates from leaves that remain green for longer), or the stay-green trait (or the presence of higher N concentrations in non-stay-green cultivars) actually initiates spikelet formation. In experiments on the shrub *Chuquiraga oppositifolia* D. Don (Asteraceae), increased floral initiation and a higher seed output resulting from supplemental N supply were only seen in the third year after application, with increased vegetative growth being seen in the second year (Muñoz *et al.*, 2005). In *A. thaliana*, which has a rosette pattern of growth, high concentrations of nitrate and carbohydrates in the rosette leaves do not contribute directly to seed filling, but maintain the capacity of the leaves to keep assimilating resources for a long time (Schulze *et al.*, 1994).

By contrast, there is evidence that in normal field-grown wheat crops with good N supply the yield obtained is sink-limited and not source-limited. Spring wheat transformed with a leaf rust gene from *Agropyron elongatum* (Host.) P. Beauv. plants had 11% more grains per spike than wild-type plants, but each grain weighed only 3.5% less, and total plant biomass was 9% higher (Reynolds *et al.*, 2005). The plants showed increased photosynthesis, particularly at the grain-filling stage, and Reynolds *et al.* (2005) suggested that this increase in photosynthesis came about because of the increased sink strength. This means that photosynthesis is normally down-regulated, which explains why plants that have leaves removed are able to compensate for the loss of leaf area by increased photosynthesis in the remaining leaves. In cereal plants, N fertilization is used to increase leaf area duration, and so increase source pressure, but this could actually be controlled by sink strength. In N-efficient maize genotypes, kernels seem to continue developing more when the plants are N-depleted than in N-inefficient genotypes, and this seems to maintain active, photosynthesizing leaves for a longer period (Paponov *et al.*, 2005). Determining precisely how sink strength and source pressure interact under N deficiency remains a challenge.

13.4 Partitioning of nitrogen into metabolites

It can be seen that there is partitioning of nitrogen into different plant parts under different environmental conditions, and with the ageing of plants, but is nitrogen also partitioned differently into different molecules at different stages of the life cycle and under different conditions?

In experiments in which tobacco (*N. tabacum*) transformed with antisense *rbc*S to decrease expression of ribulose bisphosphate carboxylase/oxygenase, and grown in different rates of N supply, the relative constancy of partitioning into different molecules could be seen (Fichtner *et al*., 1993). At low rates of N supply there was low ribulose bisphosphate carboxylase activity in both transformed and wild-type plants, but at high and intermediate rates of N supply there was more ribulose bisphosphate carboxylase activity per unit leaf area in both sets of plants, although the antisense plants had less ribulose bisphosphate carboxylase activity per unit leaf area than the wild types. There was a strong positive correlation between ribulose bisphosphate carboxylase activity per unit leaf area and total leaf area at high N supply, a less strong correlation at intermediate N supply and low values of both leaf area and ribulose bisphosphate carboxylase activity at low N supply. This meant that there was a greater leaf area in the high N plants, and consequently more ribulose bisphosphate carboxylase activity per plant, and there was more ribulose bisphosphate carboxylase per plant in the wild-type than in the transformed plants. Those plants with more ribulose bisphosphate carboxylase activity per unit leaf area had lower values of SLA (i.e. thicker leaves), so the amount of ribulose bisphosphate carboxylase activity per unit plant mass did not show big differences between the wild-type and transformed plants. The SLA tended to be bigger in the transformed plants than in the wild types at high rates of ribulose bisphosphate carboxylase activity, but there was no difference between the two types of plants at the low values of ribulose bisphosphate carboxylase seen with low N supply. Proportions of the components of the photosynthetic machinery involved in the light-independent reactions may vary relative to the components of the light-dependent reactions with differences in irradiance (see Section 13.6), but overall these essential metabolites seem to be present in amounts that optimize the growth of plants.

Other experiments where the amounts of key enzymes have been artificially altered include a study of transgenic poplar (*P. tremula* × *P. alba*) hybrids overexpressing cytosolic glutamine synthetase (GS), and which were 41% taller than control plants after 3 years' growth (Jing *et al*., 2004). They had increased protein, total GS and ferredoxin-dependent GS in the leaves and higher levels of vegetative storage proteins in the stems. Similarly, enhanced growth (fresh and dry mass) was seen in tobacco (*N. tabacum*) plants transformed to overexpress cytosolic GS, where growth was better in both low and high N supply (Oliveira *et al*., 2002). The important aspect of these transformations was that it was glutamine synthetase in the leaf cytoplasm

that was overexpressed, not chloroplastic glutamine synthetase, so the plants were able to efficiently reabsorb the ammonium released during photorespiration (Oliveira *et al*., 2002). The cytosolic form of the enzyme would also have an involvement in the remobilization of fixed N from leaves to other organs during senescence. The chloroplastic form of GS is most active in green, photosynthesizing leaves, and the activity of the cytosolic form increases as leaves senesce (Habash *et al*., 2001). In the experiments of Hirel *et al*. (2001), it was seen to be a positive correlation between cytoplasmic GS activity and maize plant yield, but a negative correlation between nitrate reductase activity and plant yield, and the authors showed that the plants with the highest nitrogen use efficiency had low nitrate reductase activity (and high nitrate contents of the leaves as a result) and high GS activity. The plants shared quantitative trait loci for nitrate reductase and GS activity, implying that the two enzymes are co-regulated. Obara *et al*. (2001) have shown that the alleles that increase the amount of cytosolic GS in rice leaves also help bring about leaf senescence, further indication of the importance of the enzyme in remobilization of fixed N into developing seeds. However, other studies have found less convincing effects of transforming plants to express cytosolic GS and no effects on plant growth of transforming other enzymes associated with N metabolism (Good *et al*., 2004).

We have already seen that N supply can alter the expression and activity of enzymes of C_3 and C_4 photosynthesis and the Krebs cycle (Yamazaki *et al*., 1986; Lancien *et al*., 1999; Pasqualini *et al*., 2001; Foyer *et al*., 2003). In addition, many of the enzymes of nitrogen assimilation are controlled by ammonium and nitrate, either at the level of expression or post-transcriptional control or both.

Partitioning of nitrogen into secondary metabolites is affected by its availability. For example, supplying plants with high rates of nitrogen can increase concentrations of alkaloids (Nowacki *et al*., 1976). In tobacco, the major alkaloid present is nicotine, and concentrations of nicotine were higher at higher rates of nitrate or ammonium supply (Matt *et al*., 2002). This is not surprising given that nitrogen is a component of alkaloids, but in this study plants transformed to have lower expression of ribulose bisphosphate carboxylase/oxygenase had lower nicotine concentrations per unit fresh weight than untransformed plants. The concentration of this secondary metabolite was affected by availability of fixed carbon as well as nitrogen, further evidence of the importance of the interactions between nitrogen and carbon metabolism. N deficiency in tobacco gives rise to less synthesis of nicotine and more synthesis of carbon-rich phenylpropanoids, a switch that is brought about by nitrate in the plant rather than metabolites arising from its assimilation (Fritz *et al*., 2006).

Another group of secondary metabolites that contain nitrogen are the cyanogenic glycosides. In *Eucalyptus cladocalyx* F. Muell., up to 15% of leaf nitrogen is present in the cyanogenic glycoside prunasin, but precise concentrations vary according to environmental factors (Burns *et al*., 2002). Under

shaded conditions, when carbon assimilation is more difficult, prunasin concentrations are lower relative to total leaf N content, whereas chlorophyll concentration and the CO_2 assimilation rate are higher, indicating that the nitrogen is used more for enhancing carbon assimilation under these conditions (see also Section 13.6).

The concentrations of secondary metabolites are also affected by other external factors, and these changes often influence the putative roles that we ascribe to different compounds. The proportions of plant nitrogen in nicotine in N-depleted tobacco (*Nicotiana sylvestris* L.) have been shown to be higher in plants in which the leaves have been damaged (Ohnmeiss & Baldwin, 1994), and this fits the role that we assume nicotine fulfils in protecting tobacco from herbivory (see Section 13.6). It seems that nitrogen can be partitioned into protective compounds at times when the plants are under potential attack. Why might nicotine concentrations not be high all the time if they give the tobacco plant a protective advantage, however? The nitrogen (and also the carbon) in the nicotine could be used in primary metabolism, in enzymes such as ribulose bisphosphate carboxylase for example, and tobacco genotypes with lower nicotine concentrations tend to have higher yields than genotypes with higher nicotine concentrations (Matzinger *et al.*, 1972). The increased nicotine seen in the damaged plants of Ohnmeiss and Baldwin (2000) came from synthesis in the roots using nitrogen taken up after the damage was inflicted (Baldwin *et al.*, 1994), but the nicotine is translocated to different leaves in different proportions depending on their importance in maintaining the productivity of the plant at whatever point in its life cycle the damage occurs (Ohnmeiss & Baldwin, 2000).

13.5 Acquisition of nitrogen by plants

With plant growth being so affected by N supply, in terms of the final yield of crops, partitioning of biomass into different plant parts and even into the proportions of different molecules within plants, we might expect that all aspects of plant growth are entirely dependent on the amount of nitrogen present in the rooting medium. However, the presence of plants alters the physical and chemical nature of the rooting medium, and the plants can act both directly and indirectly to make nitrogen available.

In a study of a large number of datasets for crop yields of lucerne, maize, oilseed rape, rice, sorghum, sunflower and wheat (Lemaire *et al.*, 2007), it was shown that plotting amount of N taken up by the crop per land area against the crop biomass per land area gave curves that closely fitted what could be predicted for the N uptake based on previously known values of critical N content in the plants at each of the different biomass values. This indicates that in a series of crops grown in different experiments the individual plants contained close to their critical N contents irrespective of whether they were in high-yielding, low-yielding or intermediate crops. There was no

obvious luxury uptake of N, but also no indication of many situations where crops were obviously N-deficient. Therefore, although we know from classical experiments and agricultural practice that crop yields can be increased by N fertilizer application, over entire growing seasons N uptake is actually feedback-regulated by the ability of the crop to accumulate biomass (i.e. uptake is regulated by demand).

Nitrate is freely water soluble, and in field-grown crops and uncultivated plants, it can be transported to the root surface by mass flow. At high availability of nitrate (~7 mM in the soil solution), mass flow can theoretically move enough nitrate to the root system for plant growth, and its rate of uptake is then dependent purely on the rate of uptake across the root surface (Engels & Marschner, 1995). However, at lower concentrations, insufficient nitrate would move to the root surface, and diffusion is important; the rate at which diffusion of nitrate occurs across the rooting environment limits the rate of uptake.

Ammonium salts are much less mobile in soil, being less soluble, and the NH_4^+ ion is attracted to the negative charges on clay colloids. Therefore, the presence of ammonium ions at the rhizoplane is due much more to diffusion than to mass flow, but the ion has a lower diffusion coefficient than nitrate.

For uptake of both nitrate and ammonium, a plant has various carriers and ion channels, the behaviour of which can be altered by both presence of more (or fewer) of them and modification of kinetic parameters (e.g. Glass *et al.*, 2001). In experiments on individual plants over short periods we can see induction of uptake mechanisms, both carriers and channels for nitrate and ammonium, which indicates the potential for plants to take up ever-increasing amounts of nitrogen as uptake is induced, giving rise to more nitrogen assimilation and more plant growth, and so a larger root system for even more uptake. The rate of uptake of nitrate or ammonium per plant is dependent on both the specific activity of the root tissues and the size of the root system. The rate of nitrate uptake per unit root dry weight increases with increase in root N concentration, up to an optimum value, above which it becomes lower with further increase in root N (Mattsson *et al.*, 1991). Increasing N concentration of the root from low values could give rise to more carriers and channels, enhancing N uptake, but N concentrations above the optimum value may only occur because of lack of demand from the plant for additional nitrogen, so uptake may be subjected to feedback repression. At low rates of N supply, when internal N concentrations in the roots are low, the plant has a larger root–shoot ratio, so the proportionally larger root system allows more uptake to occur (Mattsson *et al.*, 1991).

For plants with adequate N supply, there is a gradual decrease in root–shoot ratio during vegetative growth. We would expect the proportion of biomass allocated to roots to be optimal for maximum uptake of nitrate or ammonium to give the amount of shoot growth, and hence leaf area, for optimum utilization of the photosynthetically active radiation available. In plants suffering a shortage of nitrogen, in experiments frequently an imposed shortage applied

to previously N-replete plants, there is an obvious increase in root–shoot ratio in plants with sufficient N. This gives the plant the opportunity to maintain root growth, so increasing root surface area, and the volume of soil explored, whilst maintaining comparatively high shoot N concentrations through avoiding too much dilution of this by excessive shoot growth.

Nitrate uptake per unit root weight increases throughout vegetative growth, and then declines during reproductive growth (Imsande & Touraine, 1994), so that the shoot is able to grow proportionally larger because of an increase in efficiency of N uptake. Furthermore, actual uptake of nitrate is usually lower than the potential uptake (Imsande & Touraine, 1994), so there is spare capacity for uptake within the root system.

Uptake of any ion is the excess of influx over efflux, and the rate of nitrate uptake by diffusion should be dependent on the difference in concentration of the ion one side of the plasmalemma to the other. For a piece of root, uptake of nitrate by means of carriers can occur against the concentration gradient, but for uptake through channels, a high internal NO_3^- concentration would lower uptake by either increasing efflux or decreasing influx, or both. In spinach plants grown in two different concentrations of nitrate, both of which were high enough that plant RGR was not different between the two treatments, nitrate influx and efflux and net nitrate uptake rate (NNUR, rate of uptake of nitrate per unit plant weight) were independent of external nitrate concentration, so uptake of the ion must have been regulated by internal factors (Ter Steege *et al.*, 1999). Plants with higher RGR had lower nitrate influx and efflux rates, but there were no significant differences in NNUR.

In a study of different wheat cultivars there were big differences in the rates of nitrate uptake per unit root weight between the cultivars, but internal N concentrations in roots and shoots were very similar, so there appears to be a level of homeostasis in internal N concentration and net nitrate uptake is not directly dependent on this (Rodgers & Barneix, 1988). In this experiment the rate of uptake per unit root weight was positively correlated with shoot RGR. In the experiments of Ter Steege *et al.* (1999) the plant nitrogen concentration was very similar between the high and low rates of nitrate supply, and by multiplying the value for internal nitrogen concentration by RGR the authors calculated the N demand of the plants. Plants with higher rates of RGR had a higher N demand (as internal N concentration was similar between plants), but the demand was reasonably well satisfied by the rate of net nitrogen uptake. This meant that the rate of uptake matched the plant demand for nitrogen, showing that under steady-state conditions nitrate uptake is demand-led.

Given that the size of the root system and its activity are optimal for the overall size of the plant in the environmental conditions under which it is growing, there is still ability for different parts of the root system to show different levels of activity to cope with differences in availability of nitrogen in different parts of the root zone. In experiments on *B. napus* plants with split roots, depriving one part of the root system of nitrate resulted in nitrate

uptake rate increasing in the other half of the root system within 24 hours (Lainé *et al*., 1995). This increase in uptake rate seemed to be brought about by a cellular signal from the shoot. It is clear from split-root experiments that plants take up sufficient nitrate from the halves of the root systems supplied with nitrate to compensate for the halves of the root systems that are not supplied nitrate, so that the growth of the plant is not appreciably different when nitrate is supplied more evenly across a root system (Öhlén & Larsson, 1992). In experiments in which maize plants were grown in different root zone and shoot base temperatures, and with either nitrate N or ammonium N, there was a linear relationship between the rate of translocation of N from roots to shoots and the demand placed on the roots by shoot growth (Engels & Marschner, 1996). Plants that had shoots with low demand for nutrients were generated by keeping the shoot base temperatures low, and the rate of net translocation was higher in those plants with high demand shoots. This further indicates that uptake is directly dependent on the demand the shoots place on the roots for supply of nutrients (Engels & Marschner, 1995, 1996).

In maize seedlings grown in low root zone temperature/low shoot base temperature, low root zone temperature/high shoot base temperature, high root zone temperature/low shoot base temperature, high root zone temperature/high shoot base temperature, both the high shoot base temperature treatments gave a faster rate of uptake of nitrate N per unit root mass irrespective of root zone temperature (Engels & Kirkby, 2001; Fig. 13.3). It is clear that the demand placed on the root system by the shoots gave more efficient use of the uptake systems in the plants in which shoot growth was not constrained by low temperature. Even in the plants with low shoot base temperature and high root zone temperature, the rate of N uptake per unit root mass was low, indicating that even the energetically favourable conditions inside the root did not allow the uptake systems to function at their potential rate. However, in these plants there was a much higher rate of growth of roots relative to shoots than in the other treatments, so despite this low level of efficiency of the uptake systems, the plants still acquired sufficient nitrogen that the rate of accumulation of biomass of the roots and shoots together was not much lower than the rate of accumulation of total biomass in the plants with low root zone temperature and high shoot base temperature.

If nitrate uptake is regulated by demand for plant growth, how is this sensed at the root epidermis plasmalemma? Plants seem to be able to signal their nitrogen status by the movement of amino acids in the phloem (Cooper & Clarkson, 1989), and the amino acid composition of the phloem sap seems to exert feedback control over nitrate uptake (Imsande & Touraine, 1994; Gessler *et al*., 1999). In slower-growing plants, there is presumably proportionately more unloading of amino acids in the roots, and less net uptake of nitrate. In the plants shown in Figure 13.3, a higher proportion of N taken up moves from shoots to roots in the plants grown with high root zone temperature and low shoot base temperature than in the plants in the other three treatments, which is presumably one of the reasons why rate of uptake per unit root mass

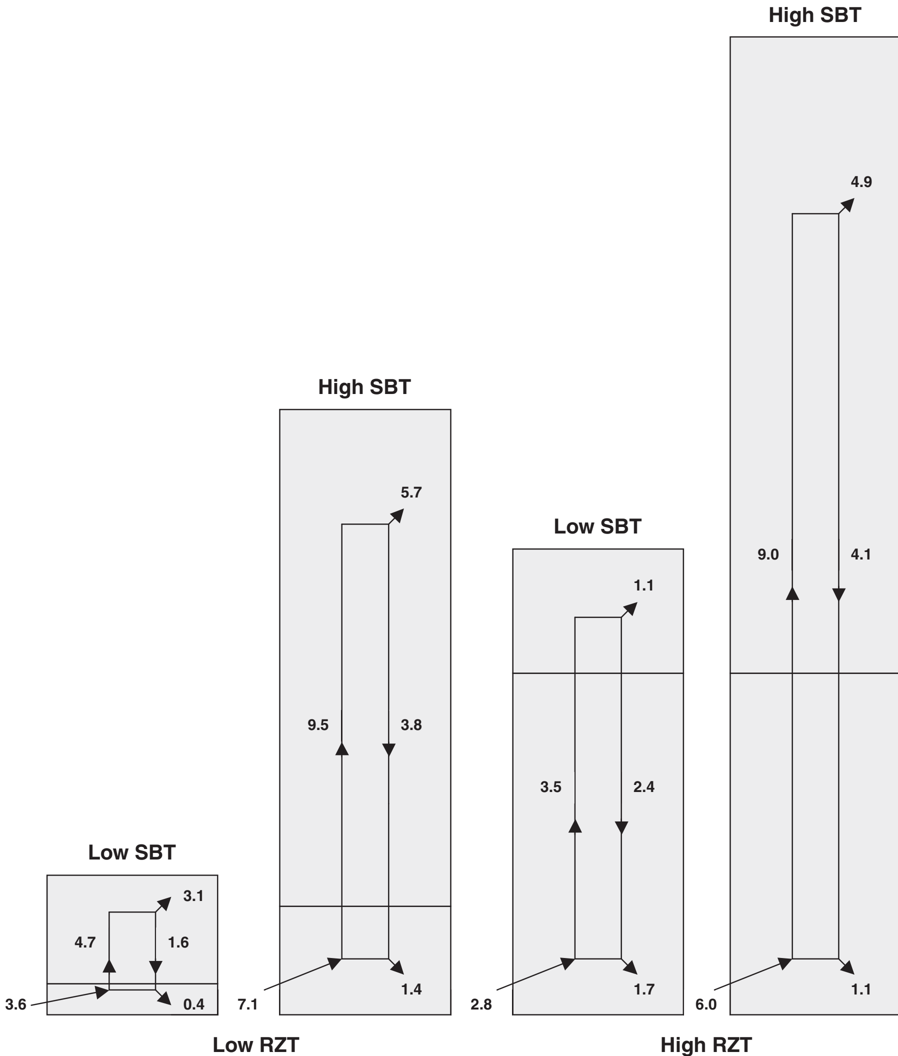

Figure 13.3 Uptake and cycling of nitrogen in young maize plants subjected to different shoot and root temperatures. Maize (*Zea mays*) plants were grown in root zone temperatures (RZT) of 12° (low) and 24°C (high) and shoot base temperatures (SBT) of 12° (low) and 24°/20°C day/night (high). Boxes are approximately to scale in relation to the rate of increase in mass of shoots and roots, and numerical values are of N flux per unit root mass (μmoles/hour/(g root fresh weight)$^{-1}$). (Based on data in Engels & Kirkby, 2001. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Data shown reproduced with permission.)

is lower in these plants (Engels & Kirkby, 2001). However, there may be other shoot-derived signals involved as in *R. communis* plants grown in split-root experiments withdrawing nitrate from one half of the root system led to a faster rate of nitrate influx in the other half of the root system, without there being any obvious changes in amino acid profile in the phloem directed to those roots (Tillard *et al.*, 1998).

Where nitrate is the N form available to plants, diffusion becomes increasingly important at low rates of supply. The rate of diffusion is dependent on the difference in nitrate concentration between the bulk soil and at the root surface, the water content of the soil and the root surface area, and so root length, root hair density, root hair length and root diameter can also be modified to facilitate continuing nitrate uptake (Robinson, 1994, 1996, 2001). At high availability of nitrate, the plant needs to maintain transpiration to keep the ion moving to the root surface by mass flow. Where ammonium is the N source, the plant needs to maintain the structure of its root system to maximize diffusion. New roots that grow during periods of both N sufficiency and N deficiency have uptake systems that are optimized for the concentrations of the nitrate and ammonium ions at the plasmalemma of the epidermis, and the uptake systems in the older, but still functional, parts of the root system can adapt due to mechanisms that rapidly signal the nutrient availability.

The availability of nitrate also affects the ability of the root system to take up water. Roots have low hydraulic conductance when the availability of nitrate is low (Clarkson *et al.*, 2000), but supply of nitrate to sunflower plants grown in low nitrate led to a rapid increase in hydraulic conductance of the roots (a response apparent within 20–30 minutes) and an increase in the xylem flow rate (Gloser *et al.*, 2007). This increase in hydraulic conductance was not due to the increased presence of nitrate in the roots as inhibition of nitrate reductase activity in the roots prevented it from occurring. However, other authors have shown a very rapid increase in hydraulic conductivity of roots upon exposure to nitrate that is apparent at the cell membrane level, and which does seem to be brought about by intracellular nitrate (Gorska *et al.*, 2008). It seems possible that the uptake of nitrate may cause expression of aquaporins, channels through which water uptake into roots occurs (Clarkson *et al.*, 2000; Gloser *et al.*, 2007), although whether this expression comes about from a product of nitrate assimilation or from the process of assimilation itself (Gloser *et al.*, 2007), or from nitrate inside the root (Gorska *et al.*, 2008) is less certain. Ammonium suppresses aquaporin expression, and in split-root experiments on *Phaseolus vulgaris* L. plants the halves of the root systems supplied with nitrate had much higher water uptake than the halves supplied with ammonium, or no nitrogen at all (Guo *et al.*, 2007).

Increased uptake of water in the presence of nitrate would permit more transpiration to occur and so enhance mass flow both of the water and the nitrate dissolved in it. In effect, as roots grow into areas of soil containing nitrate, this could not only induce nitrate uptake mechanisms but also facilitate the movement of more nitrate to the root surface in the soil water.

Ammonium uptake occurs through high-affinity carrier mechanisms and low-affinity mechanisms that may be channels (Glass *et al*., 2001), possibly including potassium channels and non-selective cation channels (Kronzucker *et al*., 2001). There is a high-affinity uptake system that works less well when internal N concentrations are high, and a low-affinity system that does not seem to be affected by abundant ammonium supply (Glass *et al*., 2001). It is also possible that uptake of ammonium may occur through mycorrhizal associations (Ames *et al*., 1983), and in experiments in which *Plantago lanceolata* L. was grown with the arbuscular mycorrhizal fungi *Glomus hoi* or *Glomus intraradices* that were able to access dried leaf material containing ^{15}N and ^{13}C, a large proportion of the N (but not the C) in the patch was transferred to the plants, and contributed a high proportion of total N acquired by the plant (Leigh *et al*., 2009). Plants grow better with a mixed nitrogen supply (Cox & Reisenauer, 1973), but for this benefit to be seen the nitrate and ammonium should be supplied evenly and not to separate parts of the root system (Schortemeyer *et al*., 1993). Uptake of ammonium is stimulated by the presence of nitrate at the part of the root system where the uptake occurs (i.e. it does not seem to be dependent on a shoot-generated signal) (Saravitz *et al*., 1994). This may help plants avoid ammonium toxicity, and in plants supplied with a low ratio of nitrate–ammonium there is stimulation of activity of the high-affinity nitrate transporter although low concentration of nitrate *per se* might be expected to give a low rate of activity of this transport mechanism (Krouk *et al*., 2006).

There is an increasing interest in the uptake of amino acids by plants (Fischer *et al*., 1998; Näsholm *et al*., 2009), which tends to be overlooked in the study of nitrogen nutrition. Estimates for different plant species indicate that anywhere between 10 and 90% of a plant's N requirement might come through uptake of amino acids from the soil (Lipson & Näsholm, 2001). Uptake of amino acids may occur through mycorrhizal associations (Tibbett *et al*., 1998), and it is also possible that mycorrhizal fungi may facilitate the movement of nitrogen from one plant to another (Moyer-Henry *et al*., 2006). In field experiments on maize intercropped with common bean (*P. vulgaris*), there was indirect evidence of transfer of nitrogen from the nodulated bean plants to the maize, presumably through mycorrhizal connections (Dawo *et al*., 2009).

As well as there is control of the influx and/or efflux of nitrate and ammonium, the roots adapt structurally to the rate of N supply. There are four major effects of nitrogen supply on root system structure, namely, a local stimulation of lateral root elongation caused by localized high nitrate supply, an inhibition of lateral root meristems by high tissue nitrate concentration, a suppression of lateral root initiation caused by high C–N ratios in the tissues and inhibition of primary root growth and stimulation of root branching by external supply of glutamic acid (Zhang *et al*., 2007). Additionally, the density of root hairs is higher at low concentrations of external nitrate (Föhse & Jungk, 1983).

We know that the soil environment is extremely heterogeneous in concentrations of available nitrogen, both between different ecosystems and between small distances within an ecosystem. Concentrations of soil nitrate can vary by several orders of magnitude within natural ecosystems (Jackson & Caldwell, 1996; Glass *et al.*, 2001), yet in mature forests nitrate can be almost undetectable in soils, with ammonium being the predominant N form (Glass *et al.*, 2001). Even in an agricultural environment, soil nitrate and ammonium concentrations are unlikely to be homogeneous as there will be patches of high availability from uneven fertilizer applications. It can therefore be seen that the adaptations of uptake mechanisms and root architecture should be able to optimize acquisition of nitrogen from patchy environments, and modelling exercises have certainly shown that increased root density and up-regulation of uptake mechanisms in patches of high availability of nitrate give enhanced uptake of nitrate from these patches (Jackson & Caldwell, 1996). However, change in root density could be a relatively long-term effect, and plants may respond to their roots growing into nitrate-rich patches by very rapid increase in hydraulic conductivity in the part of the root system affected, as referred to earlier (Gorska *et al.*, 2008). This suggestion is strengthened by the observation that in split-root experiments the higher conductivity towards water of the part of the roots exposed to nitrate is rapidly matched by a lower conductivity in the rest of the root system, indicating that there is a rapid signalling system for the enhanced uptake of water (and the nitrate dissolved in it) in one part of the root system that could possibly be a change in the water potential gradient (Gorska *et al.*, 2008).

Agronomists think of the importance of nitrogen (and other elements) to plants in terms of Mitscherlich curves for responses of plants in terms of their growth or yield to availability of individual nutrients, and this is the basis of fertilizer recommendations in agriculture. However, ecologists tend to consider plant growth in terms of the multiple limitation hypothesis (Bloom *et al.*, 1985; Gleeson & Tilman, 1992). This hypothesis puts forward the idea that plant growth is limited by many constraints simultaneously, so that the morphology and physiology of a plant at any one time are adapted to optimizing the acquisition of resources required for growth in the most beneficial proportions. The multiple limitation hypothesis is stated to be incompatible with Sprengel's and Liebig's Law of the Minimum as in the former a plant can trade-off one resource in abundant supply to acquire another resource in limited supply, whereas Sprengel and Liebig thought that growth is limited by whichever nutrient has most limiting supply relative to the plant's requirements (Rubio *et al.*, 2003). For example, a plant with adequate supplies of carbon fixed in photosynthesis can use this resource for increased root growth in response to a shortage of nitrogen in the first theory, but not in the second.

The suggested incompatibility between the two theories is not necessarily correct as in this example future acquisition of carbon by the plant is restricted by the increased proportion of fixed carbon going to the roots rather than to

the development of new leaves. The growth of the plant is restricted by the nutrient in most limiting supply relative to the plant's requirement for it, nitrogen, as the Law of the Minimum would indicate.

However, the multiple limitation hypothesis does show how the functional equilibrium between shoot and root optimizes a plant's ability to survive in the aerial and edaphic conditions in which it lives. It may also work better with the uptake of two mineral elements taken up through the roots, such as phosphorus and nitrogen, where an excess of one may possibly be used to increase the uptake of the other. In this example nitrogen must also be allocated more to the roots in P-deficient plants than would normally be the case, but overall N uptake by the plant is constant as there is a lower requirement for N for shoot growth. This occurs because the shortage of phosphorus limits future carbon acquisition, and there is no deficiency of N. If the photosynthetic capacity of the leaves (the source pressure) were actually higher than demand for fixed carbon for growth of new organs (sink strength), the extra root growth following from P deficiency would not be at the expense of shoot growth, so more N would be taken up. The excess of N available to it, as well as the excess of carbon, would enable the plant to increase root growth so that a larger volume of soil was explored, but it could also maintain shoot growth so that future carbon acquisition was not jeopardized. In this case the excess of N and C together would enable it to acquire more P.

In this example the plant maintains uptake of N whilst adapting to the shortage of P, so the ratio of N–P in the tissues increases until uptake of P is restored to its previous rate. The use of N to increase P uptake can therefore only occur if plants do not maintain homeostasis in N–P tissue concentration. Despite the relationship between internal N concentration and growth rate (and partitioning of dry matter into different plant parts), there is a relatively constant proportion of N in plants in relation to P, K, Ca and Mg (Knecht & Göransson, 2004). However, there is also abundant evidence of variable N–P ratios in plants. For example, Gunes *et al.* (1998) showed that the N–P ratio in tomato increases with increased N nutrition and decreases with increased P nutrition. In the grass species *Phleum pratense* L., it has been shown to be a linear relationship between internal N and P concentrations if N supply is not limiting to plant growth, but when N supplies are limiting, higher concentrations of P occur in the tissues (Bélanger & Richards, 1999). Some variability in N–P ratios in tissues can be accounted for by differences in proportions of different types of tissues, but it appears to be relatively well-defined N–P ratios in tissues related to plant growth. These ratios have been defined as being 11.83:1 in growth-related tissues of many crops, with values of 12.65:1 and 13.64:1 being seen in terrestrial and freshwater autotrophs on average (Greenwood *et al.*, 2008). The N–P ratio of whole plants declines as the plants age, along with RGR, due to the decline in proportion of growth-related tissues (Ågren, 2004; Greenwood *et al.*, 2008). At a coarse level, and considering plant yields over a growing season, the extent to which increased

uptake of N can substitute for shortage of other elements (and vice versa) must be limited. If this were not the case, farmers would not apply different ratios of N, P and K to different crops – ratios that have been well documented for many years – to obtain optimum yield of each crop. However, plants do adapt their distributions of carbon and nitrogen when one is limiting to optimize its acquisition.

13.6 Plants, nitrogen and environment

One of the responses of plants to encountering patches of nitrate in the soil is to proliferate lateral roots, although the solubility of the ion makes it freely available without such proliferation (Robinson, 1996). For crop species growing in fertile agroecosystems, there is probably sufficient nitrate present for the needs of the plants to be met by mass flow, so that adaptations of the root systems to cope with low availability of nitrate are rarely required (Engels & Marschner, 1995). However, in an experiment on fast-growing cottonwood (*Populus deltoides* Bartr.) grown in a mixture of soil and sand, and supplied $^{15}NO_3^-$, uptake of ^{15}N was strongly correlated with root mass, and to a lesser extent with transpiration per unit root mass (McDonald *et al.*, 2002). In natural ecosystems plants compete for nitrogen in most soils, and possibly root proliferation gives one plant a competitive advantage over another in terms of its ability to acquire nitrogen in a mixed stand (Hodge *et al.*, 1999). Increasing the availability of nitrogen in an ecosystem changes the competitive interactions between different plant species, and changes species composition.

Under low N supply, RGR of plants is similar despite whether or not the species normally occurs in a nitrogen-rich environment, but under high N supply those plants that are normally found under this condition have a faster RGR than plants that naturally occur in nutrient-poor environments (Fichtner & Schulze, 1992). Fast-growing species allocate more N to leaves and roots than to stems; they carry out more photosynthesis per unit of N in each leaf (they have a higher photosynthetic N use efficiency, PNUE), and as a result of these two factors, they accumulate more biomass per unit of N taken up (they have a higher N productivity) (Poorter *et al.*, 1990). Species with typically fast growth rates have short-lived leaves and higher leaf N concentrations per unit mass (Reich *et al.*, 1998; Leishman *et al.*, 2007). This gives them more metabolic activity (both photosynthesis and respiration) per unit leaf mass. NAR, the rate of increase in biomass per unit leaf area, is higher in plants that are adapted to nitrogen-rich areas, but only under abundant N supply (Fichtner & Schulze, 1992).

In a comparison of different *Poa* species with a range of growth rates, the fastest-growing species had the highest values of PNUE, and this was largely brought about by more of the leaf N being allocated to photosynthesis. The fast-growing species also had higher SLA (Westbeek *et al.*, 1999). In a comparison of fast- and slow-growing monocotyledonous species (van der

Werf *et al.*, 1993), the fast-growing species had much higher SLA and N productivity than the slow-growing species at high rates of N supply, but at low rates of N supply the values of SLA, although still higher in the species that could potentially grow faster, were more similar between the species and there were no big differences in N productivity between the two groups. The positive correlation between PNUE and the SLA of a plant species occurs because when leaves are thinner there is less use of nitrogen in non-photosynthetic metabolites (Pons & Westbeek, 2004). Values for PNUE in plants range from below 50 to above 140 μmol CO_2 $[\text{mol N}]^{-1}$ s^{-1} (Field & Mooney, 1986).

Species that tend to occur in nitrogen-rich environments typically have higher SLA (Fichtner & Schulze, 1992). In a comparison of a fast-growing grass, *Dactylis glomerata* L., and a slow-growing grass, *Brachypodium pinnatum* (L.) P. Beauv., both responded to increased N supply by decreasing their root length and root weight ratio (the proportion of roots as a proportion of plant mass), and by increasing their leaf area relative to plant mass (Ryser & Lambers, 1995). However, *D. glomerata* had a higher leaf area ratio (leaf area per total plant mass, LAR) and a higher root length ratio than *B. pinnatum* at all rates of N supply. Both species increased their SLA with increased N supply, although *D. glomerata* had higher values at each rate of N supply and increased its SLA more than *B. pinnatum*. Both species were able to respond to increased N supply by investing proportionally more of their resources into shoot growth to increase the potential for carbon assimilation, but in the case of the fast-growing *D. glomerata*, the production of thinner leaves gave a larger leaf area per unit of resource invested, thus enabling that species to have a faster growth rate.

SLA is adjusted in plants to optimize interception of sunlight, and leaves exposed to bright light have a lower SLA than leaves in shade. In fact, many species are very plastic in terms of SLA, and this can be adjusted if the light regime on an individual leaf changes. Typically, sun leaves have a higher N concentration than shade leaves. However, this is N content per leaf area as leaves that are thicker have more layers of cells, all contributing to the N content of each unit of leaf area. In experiments in which ten species of plants were grown in contrasting photon flux densities of 200 and 1000 $\mu mol/m^2$ per second, the N concentration per leaf mass was approximately the same in the two treatments (Evans & Poorter, 2001). The low-light plants had an SLA double that of the high-light plants, so they had half the N concentration per unit leaf area. The light-saturated photosynthetic rate per unit leaf mass was similar in both groups of plants, although it was much lower per unit leaf area in the low-light plants than in the high-light plants. It is therefore apparent that response of plants to low irradiance is to maintain the rate of photosynthesis per unit mass of photosynthetic tissues, but to distribute these tissues over a bigger area to maximize the interception of photons. The nitrogen is similarly distributed, as discussed earlier in relation to N distribution in canopies.

In an experiment in which tomato plants were grown in 'low' and 'high' irradiance (photon flux density of 70 and 300 μmol/m^2 per second, respectively, with the higher value being only one-sixth of bright sunlight but probably more than 50% of the photon flux density required for light saturation of photosynthesis in a C_3 plant), nitrogen supply had a big effect on plant morphology and the growth of the plants (de Groot *et al.*, 2002). Whilst the plants in the low irradiance always had higher SLA, and also a higher value of leaf area per total plant mass (LAR), increased rate of N supply gave higher values of SLA and LAR in both irradiance treatments. These increased values of SLA and LAR when more nitrogen was provided were particularly noticeable in the low irradiance treatment. The plants grown in high irradiance also increased their LAR and SLA in response to increased N supply, but not to the same extent, and they strongly increased their NAR particularly between low and medium rates of N supply.

As well as having increased leaf area relative to plant and leaf mass, plants grown in shade have a larger proportion of their leaf N partitioned into light-harvesting processes (Niinemets *et al.*, 1998; Evans & Poorter, 2001; Eichelmann *et al.*, 2005). In a study of three species in a Baltic woodland, species varying in their positions in the canopy, there was seen to be a higher proportion of the N invested in photosynthetic machinery being in the light-harvesting proteins, less in ribulose bisphosphate carboxylase. There was an even greater decrease in the amount of non-photosynthetic N in the shaded leaves compared with those in bright light (Eichelmann *et al.*, 2005). Similarly, in the tomato plants of de Groot *et al.* (2002), there was a higher ratio of chlorophyll $a + b$ to total N in the leaves in the plants grown in low irradiance than in the plants grown in the higher irradiance. Such physiological adaptations indicate why the light-saturated rate of photosynthesis per unit leaf mass was unaffected by the photon flux density in which the plants of Evans and Poorter (2001) were grown, and the concentrations of organic nitrogen per unit leaf mass in the two groups of plants were the same. The nitrogen was distributed to optimize the plants' use of the photons available, in low irradiance to maximize their interception and in high irradiance to give the optimum amount of light-harvesting centres and photosynthetic enzymes to maximize the use of the photons that were more easily intercepted (see Section 13.3.2).

For plant species growing in the wild, those with higher leaf N per unit mass have higher RGR, as there is a positive relationship between N per unit leaf mass and photosynthesis per unit leaf mass (Poorter *et al.*, 1990; Cornelissen *et al.*, 1997). Plants with higher values of leaf N concentration per unit leaf mass also tend to have higher SLA (Field & Mooney, 1986; Cornelissen *et al.*, 1997; Reich *et al.*, 1998), and in the experiments of de Groot *et al.* (2002) the supply of increased amounts of nitrogen to the tomato plants gave increased N per unit leaf mass and increased SLA. This is an important mechanism for success in competition between plant species. In a study on three grass species – *Agropyron repens* (L.) Beauv., *Poa pratensis* L. and

Schizachyrium scoparium (Michx.) Nash – growing together in an abandoned field, high levels of N fertilization gave vegetation with a greater biomass than in the low-N treatments, but even bigger LAI (Knops & Reinhart, 2000). A large part of this increase in LAI came from the increased proportion in the vegetation of the *A. repens*, the one of the three species that showed the greatest increase in SLA with increase in N supply, and the species that had the highest value of SLA at high rates of N application.

Slow-growing species are at an advantage with lower N supply as the thicker leaves that they form tend to remain metabolically active longer (Reich, 1993). The species with rapid growth rates, although having higher internal leaf N concentrations (per leaf mass) and higher photosynthetic capacity, have lower leaf lifespans (Silla & Escudero, 2004). Under conditions of low N supply, this would put them at a disadvantage.

Plants such as forbs fall into the category of plants with higher leaf N concentrations per unit mass, fast growth rates but short-lived leaves, whereas by contrast slow-growing conifers have longer lasting leaves, but leaves with smaller SLA (they are needles), lower leaf N concentration per unit mass and lower photosynthetic and respiratory activity per unit leaf mass (Reich *et al.*, 1998). However, the efficiency with which the leaf nitrogen is used also varies between species so that for any given value of N concentration per leaf mass forbs have higher dark respiration (and hence more metabolic activity) than conifers (Reich *et al.*, 1998).

The increased leaf N concentration and SLA with high nitrogen supply may not be a universal response, as in a study on cotton (*Gossypium hirsutum* L.) an increase in specific leaf weight (i.e. a decrease in SLA) was seen with increased N fertilization, a response that the authors attributed to more biomass being formed and making thicker leaves (Bondada & Oosterhuis, 2001). However, in general it seems that the ability to utilize nitrogen rapidly to make leaf area is part of the competitive ability of fast-growing species that are adapted to N-rich conditions, and this then gives them a competitive advantage over other species in intercepting light. The shading of other species by a few dominant species gives decreased plant species diversity in eutrophic environments (Hautier *et al.*, 2009).

Increased availability of N, and the consequent increase in foliar N concentrations, makes plants more nutritious for animals, and can affect herbivory. Supplying N fertilizer to the cordgrass *Spartina alterniflora* Loisel. increased foliar N concentration and increased the numbers of the planthopper *Prokelisia marginata* on the plants (Bowdish & Stiling, 1998). Brussels sprout (*Brassica oleracea* var. *gemmifera* Zenk.) plants receiving high rates of N supply had higher levels of infestation with the aphid *Brevicoryne brassicae* than plants receiving lower rates of N supply (Koritsas & Garsed, 1985). Leaf discs from tomato plants receiving high concentrations of nitrogen were preferentially chosen by two-spotted spider mites (*Tetranychus urticae*) over discs from plants less well supplied with nitrogen (Hoffland *et al.*, 2000). N supply to plants may also affect susceptibility to microbial infection, and

long-term supply of N increased the susceptibility of the apple (*Malus domestica* Borkh.) cultivar 'Golden Delicious' to *Venturia inaequalis* scab, and there was a reduced concentration of phenolic compounds in the leaves (Leser & Treutter, 2005). However, the resistant cultivar 'Rewena' remained resistant, despite also having reduced concentrations of phenolics. High nitrogen supply seems to increase the level of infection of plants by obligate parasites, such as rusts and powdery mildews, but low nitrogen supply increases the level of attack by facultative parasitic fungi such as *Alternaria* and *Fusarium* and bacterial diseases (Marschner, 1995). In necrotrophic bacteria and fungi, low N status of plants may affect the expression of pathogenicity and virulence/avirulence genes in the micro-organisms (Snoeijers *et al.*, 2000). Some authors have suggested that high leaf N concentrations arising from late application of fertilizers may make plants more susceptible to fungal diseases, whereas others have claimed that these plants should be less susceptible (Bancal *et al.*, 2008, and references therein). In a study of wheat crops over two seasons, there was no relationship between supply of N post-anthesis and susceptibility to the biotrophic fungi causing leaf rust (*Puccinia triticina*) or septoria leaf blotch (*Septoria tritici* = *Mycosphaerella graminicola*) (Bancal *et al.*, 2008). Whatever the overall effect of internal N status on susceptibility to pathogens, it certainly seems to be the case that susceptibility varies with the form of N supply (nitrate or ammonium) (Snoeijers *et al.*, 2000, and references therein).

High levels of N supply give rise to plants with higher internal N concentrations, and as a large amount of this N is present in proteins this would make the plants a better nutrient source for pathogens or herbivores. Furthermore, as the plants would have a high RGR there would be more meristematic tissue and more young cells that had not yet developed highly thickened cell walls. This ought to make nutrient-rich plants more susceptible to attack by other organisms. This is expressed in the resource availability hypothesis, which states that plants with abundant supplies of nutrients (including nitrogen) are more susceptible to grazing than plants with poor nutrition (Bryant *et al.*, 1989). In a study of two oak species, *Quercus ilex* L. subsp. *ballota* (Desf.) Samp. and *Quercus faginea* Lam., which grow in the forests and dehesa of Spain, the species found on the poorer soils (*Q. ilex*) had lower leaf loss to caterpillars than the species found typically on the deeper soils (*Q. faginea*) (Silla & Escudero, 2004). *Q. faginea* had a higher N concentration per unit leaf mass than *Q. ilex*.

Where the supply of nitrogen gave increased susceptibility of Golden Delicious apples to scab, the resource availability hypothesis was applicable. However, the increased susceptibility of apples to scab fungus also fits the carbon–nutrient balance hypothesis (Bryant *et al.*, 1983), which states that the nutritional status of plants affects the partitioning of resources into secondary metabolites, and so affects defence, as the high N plants had a decreased C–N ratio (Leser & Treutter, 2005). Increased availability of nitrogen ought to make it easier for a plant to synthesize secondary metabolites containing nitrogen,

such as nicotine, but the synthesis of phenolic compounds becomes more difficult. The increased proportion of C-rich phenylpropanoids, and the lower proportion of nicotine, under N deficiency in tobacco demonstrates the balance between acquisition of both elements (Fritz *et al*., 2006). It could be argued that increased shoot growth resulting from improved N nutrition might increase assimilatory capacity, and so increase the capacity of the plant to synthesize all secondary metabolites, but this is not true if fast-growing plants maintain a high N concentration in their leaves, as we have seen earlier.

In *Quercus serrata*, in common with other oaks, there is some defence against herbivory from tannins present in the leaves. In this species, leaf tannin concentration was shown to be negatively correlated with leaf N concentration (Hikosaka *et al*., 2005). Defoliation of seedlings gave new leaves that had a higher N concentration than control plant leaves, and a lower concentration of tannins. This would have given leaves that were less well protected, but able to make up for the loss of the photosynthetic capacity of the leaves that had been removed. Under those circumstances growth would have been a more important matter than defence for the plants. Similarly, in the *E. cladocalyx* trees mentioned earlier (Burns *et al*., 2002) the partitioning of nitrogen into chlorophyll rather than into prunasin in the shade would have facilitated the absorption of light energy rather than aiding defence, which would have been more important under those conditions. From the studies on secondary metabolite concentrations in plants discussed here, and earlier, it is apparent that nitrogen is partitioned into secondary metabolites only under conditions when they give the plant a selective advantage over and above that would be obtained by partitioning the nitrogen into photosynthetic mechanisms.

As well as nitrogen nutrition affecting the susceptibility of plants to attack by other organisms, the interaction of plants with other organisms affects nitrogen nutrition. This has already been seen in relation to formation of nicotine in tobacco plants under attack (Section 13.4). The infection of cereal plants with foliar diseases (e.g. leaf rust *P. triticina* and blotch *S. tritici*) may give rise to lower grain protein concentration, and this is linked to there being lower uptake of N post-anthesis, not to there being less remobilization of N from the leaves (Bancal *et al*., 2008).

There is a burgeoning literature on the interactions between plant nutrition and defence, and hypotheses regarding the defence of plants against herbivores and pests are well described elsewhere (e.g. Stamp, 2003; Wise & Abrahamson, 2007).

13.7 Conclusions

Careful study at the molecular level is giving us a more detailed understanding of the mechanisms of uptake and assimilation of nitrogen by plants. We are also now much more aware of how these two processes are integrated,

both with each other and with carbon metabolism, and the physiological controls of these interactions that operate.

However, we still face challenges in fully understanding the processes involved, and in particular in relating them to the growth of whole plants. Plant production in ecosystems is frequently limited by availability of nitrogen, but humankind is bringing about big changes in this respect by means of intentional nitrogen fertilization and unintentional atmospheric pollution (Vitousek *et al*., 1997). Improving productivity in agriculture will become a necessity in the face of increasing populations, not only for the amount of food that can be grown, but also in terms of the energy costs of this production. We also see a need for growing biomass and biofuel crops, and also possibly using land for carbon sequestration. In addition, we wish to protect natural ecosystems from eutrophication arising from excess nitrogen, eutrophication that can frequently alter the complex interactions between different plant species and change biodiversity. Ongoing interactions between molecular biologists, plant physiologists and ecologists will prove invaluable in helping us improve our understanding of the role of nitrogen in the growth of whole plants and crops and in ecosystems.

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INDEX

Note: Italicized *f* and *t* refer to figures and tables.

abiotic stresses, 212–13
abscisic acid, 221, 277–8
Acetobacter, 209
acidic soils, 213
actin, 150
Actinomycetales, 114
adenosine diphosphate (ADP), 214
adenosine triphosphate (ATP), 2, 210, 214
adenylate/P*i* levels, 228
ADP, 175
Aerobacter aerogenes, 189
Agrobacterium, 211
Agropyron elongatum, 323
Agropyron repens, 337–8
algae, nitric oxide synthesis, 113
alkaloids, 258–60, 324
allantoic acid, 213, 217
allantoin, 213, 217
alpha-brain waves, 172, 172*f*
Alternaria, 339
aluminium, 223–7
  nodule development, 226
  soil-borne rhizobia, 225–6
  symbiotic nitrogen fixation and, 223–5
  toxicity, 223
Amaranthus cruentus L., 317
amides, 213
amino acid permeases (AAPs), 177
amino acids, 9*f*, 174
  in bulk soil solutions, 84
  cationic transporters, 179
  encoded transporters, 177
  L-type transporters, 178
  and nitrate uptake by roots, 329
  non-protein, 174
  sequence analysis, 19
  synthesis, 289
  uptake by plants, 332
ammonia (NH_3)
  formation of, 208
  recycling, 293
ammonia assimilation, 2–23, 70–71. *See also* metabolomics; nitrate assimilation
  asparaginase, 20–23
  asparagine synthetase, 16–19
  glutamate dehydrogenase, 13–16
  glutamate synthase, 10–13
  glutamine synthetase, 2–10
  mitochondrial redox cycling, 293
  plant growth and, 307
  plant metabolomics, 255–6
  prevention, 44
ammonium (NH_4^+), 70–71. *See also* nitrate (NO_3^-)
  diffusion, 327
  formation of, 65
  metabolism, 185*f*
  in nitrogen transport, 85
  transporters, 86–9. *See also* nitrate transporters
    in peribacteroid membrane, 89
    in theanine synthesis, 182–4
    uptake by roots, 87–8
    vacuolar ammonia transport, 88–9
  uptake by plants, 332
  uptake rate, 327
AMT transporters, 86–8
AMT1 gene, 183
AMT2 gene, 183
Anabaena doliolum, 113
anoxia, 132–5, 137*f*
ANR1 gene, 276
anthocyanins, 45
aquasporin, 331
Arabidopsis thaliana, 269–83
  amino acid transporters, 178

Arabidopsis thaliana *(Cont.)*
  ammonia assimilation
    asparaginase, 21–2
    asparagine synthetase, 18–19
    glutamate dehydrogenase, 13–16
    glutamine synthetase, 3
  ammonium transporters, 183
  AMT transporters, 87–8
  antisense lines, 91–2
  brassinosteroid receptor, 112
  genome sequence, 111
  genome-wide transcript analysis, 257
  legumes and, 232
  nitrate assimilation, 70*f*
  nitrate transporters
    high-affinity transport, 91–2
    low-affinity transport, 90–91
    regulation, 92
    sensing role, 93
    uptake by roots, 90
    vacuolar, 93–4
  nitrogen transport, 86
  NO/Ca^{2+} pathways, 155
  NR regulation in, 130
  N-related morphological adaptations, 269–83
    developmental context, 274–5
    diagrams, 271*f*, 283*f*
    glutamate receptors, 279–80
    high C-N ratio-induced repression of lateral root initiation, 272, 278–9
    inhibitory effect of NO_3^- on lateral root elongation, 271, 273*f*, 277–8
    L-glutamate-induced regulation on root system, 272–4
    mechanisms, 275–80
    NO_3^- transporters, 280–81
    stimulatory effect of NO_3^- on lateral root elongation, 270–71, 272*f*, 275–7, 281–2
  NRT2 genes, 181
  pgm mutant, 257–8
  seeds and generative growth, 323
  SNF1-related protein kinases, 158
  transcriptomics, 42–4
  transforming, 27
  vacuolar ammonia transport, 88–9
Arabidopsis thaliana nitric oxide-associated 1(AtNOA1), 110
arginine pathway, 107
asparaginase, 20–23. *See also* ammonia assimilation
asparagine, 2, 16–17, 20, 213
asparagine synthetase, 16–19
aspartate, 17, 20
aspartate aminotransferase (AAT), 219
Aspergillus nidulans, 181
AtAMT2 proteins, 184
AtGC1, 111
ATH1 microarray, 43–4
AtNOS1, 110, 128
AtNRT1.1 gene, 90–91, 181–2, 276, 280–81
AtNRT1.2 gene, 91, 181–2
AtNRT1.3 gene, 91, 181–2
AtNRT1.4 gene, 91
AtNRT2 genes, 181–2
AtNRT2.1 gene, 278–81
ATP, 17, 175
autoregulation of nodulation, 211
autotrophic bacteria, 209*f*
auxin efflux carrier (AUX1), 279–80
auxin transporters (AUXs), 178
Azolla, 209*f*
Azolla pinnata, 115
Azospirillum, 209
Azospirillum brasiliense, 10, 114, 191
Azotobacter, 209, 220

Bacillales, 114
Bacillus, 114
Bacillus anthracis, 115
Bacillus subtilis, 114–15
bacterial nitric oxide synthase (bNOS), 114–15
bacteroid, 220–21
barley, nitrate assimilation in, 69*f*
bean
  leaf area index, 317
  nitrate assimilation in, 69*f*
benzyladenine, 195
beta-brain waves, 172*f*
beta-methylamino-L-alanine (BMAA), 279
Biloxi genotype, 217
biomass partitioning, 312–15
Botrytis cinerea, 153
Brachypodium pinnatum, 336
brain waves, 172, 172*f*

Brassica napus, 3, 28, 86, 91–2, 253, 319, 328–9
Brassica oleracea, 338
Brevicoryne brassicae, 338
broad bean, nitrate assimilation in, 69 *f*

C_3 plants, 316–18
C_4 plants, 69–70
Ca^{2+} channels and transporters, 150–52
Ca^{2+}-dependent protein kinases (CDPKs), 157
Ca^{2+}-mobilizing messenger, 153
caffeine, 172
callus culture, 195
Calvin cycle, 307
Camellia japonica, 173, 193, 260
Camellia sasanqua, 173, 193
Camellia sinensis, 173, 193
CaMV35s promoter, 25–7
canavanine, 174
canopy, 47 *f*, 48
  senescence, 50–53
carbon dioxide (CO_2), 75–6
  assimilation, 316
  atmospheric, 75–6
  enrichment, 75–6
  and nitrate assimilation, 69–70
  nodule permeability to, 220
carbon metabolism, 229–31
carbon restriction, 214–15
3-carboxyphenylalanine, 174
catechin, 195
Catharanthus roseus, 260
cDNA arrays, 49, 51
cereal-associative bacteria, 209 *f*
cereal-endophytic bacteria, 209 *f*
Chattonella marina, 113
chemotropism, 72
Chlamydomonas, 91, 95–6
Chlamydomonas reinhardtii, 112
Chlorella sorokiniana, 14
Chlorella spp., 113
chloroplasts, 69
Chuquiraga oppositifolia, 323
Cirsium vulgare, 312
citrulline, 108
Citrus aurantium L., 76
Clostridium, 209, 220
C-N ratios, 272, 278–9, 296–7, 339
cocklebur, nitrate assimilation in, 69, 69 *f*
constitutive high-affinity transport systems (cHATS), 90, 180
crop production, 46–8
crop yields, 326–7
cross-talk, 258–61
cyanobacteria, 65, 209 *f*
cyanogenic glycosides, 324–5
cyclic ADP-ribose (cADPR), 151, 154
Cys 3635 gene, 151
cytochrome P450, 150
cytokinin, 260–61
cytoplasmic male sterile II (CMSII), 289–93
  germination, 290
  gibberellin metabolism, 297–9
  metabolite profiling, 290–93
  mitochondrial redox cycling, 293–4
  N-sensing/signalling mutant, 295–7
  pyridine nucleotide metabolism, 294–5
  signalling, 297–9
  up-regulation of photorespiratory pathway, 294

Dactylis glomerata, 336
delta-brain waves, 172 *f*
dephosphorylation, 133
determinate nodules, 211–12
diazotrophs, 208
2,4-dichlorophenoxyacetic acid, 195
dicotyledons, 320 *f*
dinitrogen trioxide (N_2O_3), 105
6,7-dinitroquinoxaline-2,3-dione (DNQX), 279
DNA markers, 23–4
Drosera sp., 66
drought stress, 213–22. *See also* symbiotic nitrogen fixation (SNF)
  abscisic acid, 221
  bacteroid, 220–21
  carbon dioxide assimilation, 220
  carbon restriction, 214–15
  leghaemoglobin, 219–20
  nitrogen feedback regulation, 217–19
  nitrogen fixation in, 213–22
  nodule permeability to CO_2, 215–17

Encelia californica, 260
eQTLs, 54

Escherichia coli, 111, 176
ethylpyrrolidinonyl theasinensin, 175
Eucalyptus cladocalyx, 325–6, 340
expressed sequence tags (ESTs), 45, 260

FD-glutamate synthase, 190
Fd-GOGAT, 190–91
ferredoxin, 10
ferredoxin-nitrite reductase, 133
fertilizers, 63–4
  efficient use of, 46
  in tea fields, 195
Festuca arundinacea, 311, 315
flavin adenine dinucleotide (FAD), 10–11, 108, 129
flavin mononucleotide (FMN), 10–11, 190
flux analysis, 252–4
Fourier transform ion cyclotron resonance (FTICR), 252
Frankia, 209, 209*f*
fumarate, 259*f*
Fusarium, 339

gamma-amino butyric acid (GABA), 254
gamma-aminobutyric acid transporters (GATs), 178
gamma-glutamyltranspeptidase (GGT), 178–9
gas chromatography (GC), 251–2
GDH-null mutants, 192
gene expression, 53–8
generative growth, 321–3
Geobacillus, 114
germination, 139, 290
gibberellic acid, 111, 257
gibberellin, 297–9
GLN1-3 gene, 26
Glomus hoi, 332
Glomus intraradices, 332
glucose-6-phosphate isomerase, 43
glutamate, 71, 173, 185, 279–80
glutamate dehydrogenase, 13–16. *See also* ammonia assimilation
  nitrogen metabolism, 194*f*
  in plants, 191–3
  subunits, 14
glutamate receptors (GLRs), 279–80
glutamate synthase, 10–13
  GS-GOGAT pathway, 184–5, 256
  nitrogen metabolism, 194*f*
  in plants, 189–91
glutamic acid, 175, 332
glutamine, 2, 17, 53, 71, 173, 185, 185*f*, 213, 256
glutamine amidotransferase, 10
glutamine synthetase, 2–10. *See also* ammonia assimilation
  biochemical properties in plants, 185–6
  carbon restriction, 214
  in conifers, 7
  cytosolic, 324–5
  enzyme structure, 3
  expression and function of, 6*f*
  gene families, 186–7
  GS1 genes, 2–8
  GS2 genes, 2, 8–9
  GS-GOGAT system, 184–5
  inhibition, 44
  and N availability, 7
  and N metabolism, 9*f*
  nitrogen metabolism, 194*f*
  phosphorylation, 3
  phylogenetic tree of protein sequences, 5*f*
  regulation of, 187–9
  subunits, 2–3
  theanine synthesis, 178–9
glutamine:2-oxoglutarate amidotransferase (GOGAT), 10, 71, 189
glutathione reductase, 150
glycine, 256, 313
Glycine max, 224*t*, 229*t*, 317
glycosides, 324–5
Gossypium hirsutum, 338
grain expression, 49–50
gravitropism, 72
green tea, 172
GS1 gene, 2–8, 25–7, 186–9
GS2 gene, 2–10, 25–7, 186–9
GS-GOGAT system, 184–5
guanosine 3′,5′-cyclic monophosphate (cGMP), 108, 110, 115–16, 149–52
guanosine 5′-triphosphate (GTP), 108
guanylate cyclase, 108, 110

guanylate cyclase-activating enzymes, 107

Haber-Bosch process, 208
haeme-Fe, 129
haemeperoxidases, 117
HAS1 gene, 18–19
HAS1.1 gene, 18–19
HAS2 gene, 19
Helianthus annuus, 18–19
herbivores, 338–40
heterophic bacteria, 209*f*
high-affinity transport systems (HATS), 90–92, 180, 182
high-performance liquid chromatography (HPLC), 252
homeserine, 174
Hordeum vulgare, 111
horseradish peroxidase, 117
hydrogen peroxide (H_2O_2), 106
hydrotropism, 72
hydroxylamine-oxidation, 128
hydroxylamines, 128
hydroxysuccinamic acid, 20
hypersensitive response (HR), 139

impatiens, nitrate assimilation in, 69*f*
indeterminate nodules, 211–12
indo-3-butyric acid, 195
indole alkaloids, 260
inducible high-affinity transport systems (iHATS), 90, 180
iNOS, 110, 128
inosine monophosphate (IMP), 185*f*
inositol 1,4,5-triphosphate (IP_3) receptor, 150–52
introgression line (IL), 250
isoaspartyl peptides, 23
isotopic labelling, 253–4
isotopic pulse, 253

Jackson genotype, 217

Klebsiella, 220
Krebs cycle enzymes, 318
Kyoto Encyclopedia of Genes and Genomes (KEGG), 255

lactoperoxidase, 117
Lactuca sativa, 309, 310*f*
L-arginine, 128
lateral root
  density, 73*f*
  high C-N ratio-induced repression of initiation, 272, 278–9
  inhibitory effect of NO_3^- on elongation, 271, 273*f*, 277–8
  length, 73*f*
  morphological adaptations in *Arabidopsis* roots, 275–6
  stimulatory effect of NO_3^- on elongation, 270–71, 272*f*, 275–7, 281–2
Law of the Minimum, 333–4
leaf area index (LAI), 317, 319, 321, 338
leaf growth, 315–20
  carbon dioxide assimilation, 316
  low N supply, 315
leaves
  NO emission from, 130–32
  pyridine nucleotide metabolism in, 294–5
leghaemoglobin, 219
legumes, 210–12, 232–3. *See also* symbiotic nitrogen fixation (SNF)
  abiotic stresses, 212–13
  biology, 232–3
  carbon restriction, 214–15
  drought stress
    abscisic acid, 221
    bacteroid, 220–21
    carbon dioxide assimilation, 220
    carbon restriction, 214–15
    leghaemoglobin, 219–20
    nitrogen feedback regulation, 217–19
    nitrogen fixation in, 213–22
    nodule permeability to CO_2, 215–17
  genetic breeding, 233–6
  nitrogen and, 210–12
  nitrogen cycle, 209*f*
  nitrogen feedback regulation, 217–19
  nitrogen fixation under drought stress, 213–22
  nodule permeability to CO_2, 220
  nodule permeability to O_2, 215–17
  nodule structures, 211–12
  phosphate deficiency, 227–32

legumes *(Cont.)*
adenylate/P*i* levels, 228
carbon metabolism, 229–31
nodulation, 227–8
oxygen diffusion, 231–2
soil acidity, 222–7
aluminium and nodule development, 226
aluminium and soil-borne rhizobia, 225–6
aluminium and symbiotic nitrogen fixation, 223–5
aluminium toxicity, 223
leucine-rich repeat receptor-like kinases (LRR-RLKs), 112
L-glutamate, 272–4
L-glutamine, 173
liquid chromatography (LC), 251–2
L-malic acid, 176
L-NAME, 113
Lolium perenne, 281
Lotus corniculatus, 188, 188*f*
Lotus japonicus, 211, 233
low-affinity transport system (LATS), 90–91, 180
L-type amino acid transporters (LATs), 178
Lupinus albus, 229*t*
Lupinus angustifolius, 21, 229*t*, 230
Lupinus arboreus, 20
Lupinus corniculatus, 27
Lupinus luteus, 21–2
Lupinus polyphyllus, 20
Lupinus sp., 69
Lycopersicon esculentum, 308
lysine/histidine transporters (LHTs), 177

MADS box gene, 43
magnesium, in soil, 222–3
maize
AtGC1 homologue, 111
GS genes, 8
leaf growth, 319
nitrate assimilation in, 69*f*
nitrate uptake by roots, 72–5, 329
nitrogen and growth, 308
nitrogen uptake and cycling, 330*f*
transforming, 27
major facilitator superfamily (MFS), 180
malate, 213
malate dehydrogenase (MDH), 223, 230, 256
Malus domestica, 339
manganese, in soil, 222–3
mass spectrometry, 251–2
mass spectroscopy (MS), 251–2
Medicago sativa, 12
Medicago truncatulla, 3, 7, 211–12, 233, 234*f*
metabolites
partitioning nitrogen into, 324–6
profiling, 290–93
metabolome, 44
metabolomics, 251–2
ammonia assimilation, 255–6
analytical platforms, 254–5
cross-talk between N and secondary metabolism, 258–60
isotopic labelling, 253–4
metabolic steady state and flux analysis, 252–4
N incorporation into primary metabolism, 256–8
nitrate assimilation, 255–6
metal nitrosylation, 148–9
Methanocaldococcus jannaschii, 208
methionine sulphoximine, 44, 184–5
methyl jasmonate, 258
4-methylene glutamine, 174
microarrays, 43
Miscanthus, 66
mitochondrial redox cycling, 293–4
mitogen-activated protein kinases (MAPK), 155–7
Mitscherlich curves, 307, 333
Mn superoxide dismutase, 150
molybdenum-molybdopterin (Mo-MPT), 129
Monsi-Saeki equation, 317
morphological adaptations, N-related, 269–83
developmental context, 274–5
diagrams, 271*f*, 283*f*
glutamate receptors, 279–80
high C-N ratio-induced repression of lateral root initiation, 272, 278–9
inhibitory effect of NO_3^- on lateral root elongation, 271, 273*f*, 277–8

L-glutamate-induced regulation on root system, 272–4
mechanisms, 275–80
NO_3^- transporters, 280–81
stimulatory effect of NO_3^- on lateral root elongation, 270–71, 272*f*, 275–7, 281–2
multiple limitation hypothesis, 333–4
mychorrhizae, 66–7
Mycobacterium, 114
Mycosphaerella graminicola, 339
myeloperoxidase, 117

N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide hydrochloride (W-7), 157
NADH (nicotinamide adenine dinucleotide), 10, 108, 189–90, 294–5
NADH dehydrogenase, 290
NADH-GDH, 192–3
NADH-GOGAT, 12–13, 191
NADPH (nicotinamide adenine dinucleotide phosphate), 128
NADPH-GDH, 192
NADPH-glutamate synthase, 10, 11*f*, 12, 191
NADPH-GOGAT, 191
NAMB-1 gene, 49
net assimilation rate (NAR), 319
net nitrate uptake rate (NNUR), 328
Neurospora crass, 112
NHI (nitrogen harvest index), 42*t*, 46–8, 47*f*, 57*f*, 59
Nicotiana benthamiana, 140
Nicotiana plumbaginifolia, 14–15, 153–4
Nicotiana sylvestris, 287–99
cytoplasmic male sterile II, 289–93
germination, 290
gibberellin metabolism, 297–9
metabolite profiling, 290–93
N-sensing/signalling mutant, 295–7
pyridine nucleotide metabolism, 294–5
signalling, 297–9
up-regulation of photorespiratory pathway, 294
lateral root formation in, 296
mitochondrial redox cycling, 293–4
plant nitrogen in nicotine, 326
wild-type, 290
Nicotiana tabacum, 14, 88, 111, 131*f*, 154, 159, 313, 324
nicotinamide adenine dinucleotide (NADH), 10, 108, 189–90, 294–5
nicotinamide adenine dinucleotide phosphate (NADPH), 128
nicotine, 325–6
ninhydrin, 173
nitrate (NO_3^-), 67–70. *See also* ammonium (NH_4^+)
acquisition in leaves, 68*f*
assimilation, 67–70
assimilation as function of light intensity, 70*f*
diffusion, 327, 331
inhibitory effect on lateral root elongation, 271, 273*f*, 277–8
in nitrogen transport, 84–5
plant growth and, 309–11
root and shoot metabolism, 68–9
stimulatory effect on lateral root elongation, 270–71, 272*f*, 275–7, 281–2
storage pools, 309–11
uptake rate, 327–8
nitrate assimilation, 67–70, 118*f*. *See also* ammonia assimilation
mitochondrial redox cycling, 293–4
plant growth and, 307
plant metabolomics, 255–6
nitrate efflux, 90, 94–5
nitrate reductase (NR), 128–30
basic function, 128–9, 129*f*
effect of nitric oxide on, 136–8
nitrate assimilation, 256
nitric oxide formation, 107, 127
nitric oxide formation *in vitro*, 130–35
nitric oxide formation *in vivo*, 135–6
nitrogen metabolism, 257
one-electron transfer reactions, 129–30
phosphorylation, 133
physiological effects of NR-derived NO, 136–40
effect of NO on NR, 136–8
germination, 139
NO in stomatal movement, 138–9
pathogen defence, 139–40

nitrate reductase *(Cont.)*
seed dormancy, 139
senescence, 140
regulation, 130
structure, 128–9, 129*f*
nitrate transporters, 89–95
feedback and regulation, 92–3
high-affinity transport, 91–2
low-affinity transport, 90–91
nitrate efflux, 94–5
in N-related morphological adaptations, 280–81
N-responsive genes, 43
sensing role, 93
steps and mechanisms, 85*f*
thianine, 180–82
uptake by roots, 90
vacuolar, 93–4
nitrate/nitrite permeases (NNP), 89
nitrate-nitrite porter (NNP), 180
nitric acid (HNO_3), 64–5
nitric oxide, 103–18
algal nitric oxide synthesis, 113–14
chemical stability, 105
comparative NO-related signalling, 110–12
emission from leaves, 130–32
half-lifetime, 105
lifetime of, 105–6
light/dark modulation of emission, 132
mammalian-type NOS, 108–10
nitrate reductase-derived, physiological effects, 136–40
effect of NO on NR, 136–8
germination, 139
NO in stomatal movement, 138–9
pathogen defence, 139–40
seed dormancy, 139
senescence, 140
nitric oxide cycle in field, 104*f*
NO-dependent signalling systems, 106–8
oxidation, 105–6
oxidative formation, 128
production by nitrate reduction *in vitro*, 135–6
production by nitrate reduction *in vivo*, 130–35
reductive formation, 127–8
signal transduction in plants, 115–17
stimulation of emission by anoxia, 132–5
nitric oxide signalling, 147–63
in animals, 110–11, 148–52
Ca^{2+} channels and transporters, 150–52
metal nitrosylation, 148–9
S-nitrosylation, 149
tyrosine nitration, 149–50
mammalian-type, 111–12
NO/ROS interaction, 158–62
candidate sites, 159–61
impacts on HR, 158–9
protective molecule, 159–61
overview, 106–8
in plants, 111–12, 152–8
Ca^{2+}-dependent protein kinase, 157
Ca^{2+}-mobilizing messenger, 153
intracellular Ca^{2+}, 152–3
MAPK pathway, 155–7
mechanisms, 153–4
NO and protein kinases, 155
NO/Ca^{2+} pathways, 154–5
SNF1-related protein kinases, 158
nitric oxide synthase, 108
bootstrap consensus maximum parsimony tree, 109*f*
endothelial isoform, 108
mammalian-type, 108–10
neural isoform, 108
in oxidative NO formation, 128
in plant-associated bacteria, 114–15
nitrite, 127–8
in nitrogen transport, 84–5
nitrite pathway, 106–7
nitrite reductase (NiR), 256
nitrite-nitrite porter (NNP), 180
nitrochalk, 307
nitrogen, 2, 63–4
acquisition by plants, 326–35
availability in environment, 64–5
and crop production, 46–8
environment and, 335–40
forms available to plants, 83–4
mineral, 67–71
partitioning into metabolites, 323
plant growth and, 306–11

leaf growth, 315–20
photosynthesis, 317–18
seeds and generative growth, 321–3
shoot-root partitioning, 312–15
nitrogen assimilation, 1–28
ammonia assimilation, 2–23, 70–71
asparaginase, 20–23
asparagine synthetase, 16–19
glutamate dehydrogenase, 13–16
glutamate synthase, 10–13
glutamine synthetase, 2–10
mitochondrial redox cycling, 293
plant growth and, 307
plant metabolomics, 255–6
controlling, 44
crop performance and, 25–8
DNA markers, 23–5
mineral nitrogen, 67–71
nitrate, 67–70
nitrate assimilation, 67–70, 118*f*
mitochondrial redox cycling, 293–4
plant growth and, 307
plant metabolomics, 255–6
nitrogen in environment, 64–5
plant growth and, 72–5
nitrogen availability, 269–70
nitrogen cycle, 65*f*
biological elements, 209*f*
nitrogen derived from atmosphere (NDFA), 223
nitrogen fertilizers, 63–4
efficient use of, 46
in tea fields, 195
nitrogen fixation, 64–5
agricultural systems, 209–10, 209*f*
aluminium and, 223–5
nodule development, 226
soil-borne rhizobia, 225–6
aluminium toxicity, 223
biological, 209*f*
carbon restriction, 214–15
drought stress, 213–14
abscisic acid, 221
bacteroid, 220–21
leghaemoglobin, 219–20
generalized scheme, 218*f*
legumes in, 210–12
natural systems, 209*f*
nitrogen feedback regulation, 217–19
nodule permeability to O_2, 215–17
phosphate deficiency, 227–32
adenylate/P*i* levels, 228
carbon metabolism, 229–31
nodulation, 227–8
oxygen diffusion, 231–2
soil acidity, 222–3
nitrogen gas (N_2), 208
nitrogen harvest index (NHI), 42*t*, 46–8, 47*f*, 57*f*, 59
nitrogen limitation adaptation (NLA), 45
nitrogen metabolism, 23–8
and crop performance, 25–8
markers, 23–5
primary, 256–8
secondary, 258–61
nitrogen monoxide. *See* nitric oxide
nitrogen recycling, 313
nitrogen uptake efficiency (NUpE), 42*t*, 46–8, 47*f*, 54
nitrogen use efficiency (NUE), 53–8
definitions, 42*t*
measurement, 24
transcriptomics, 42–6
variations, 54
nitrogen utilization efficiency (NUtE), 46–8, 47*f*
canopy senescence, 50–53
in crop plants, 49–53
definition, 42*t*
effect of nitrogen input and genotype interactions, 55*f*
grain expression, 49–50
sink processes, 49–50
source processes, 50–53
variations, 54
in wheat, 57–8, 57*f*
nitrogen-fixing bacteria, 66
Nitrosomonas eutropha, 104*f*
nitroso-peroxocarboxylate, 149
nitrosothiols, 137*f*
Nitrosospira briensis, 104*f*
nitrosylation, 149–50
3-nitrotyrosine, 106
N-methylputrescine, 259
N-methyltransferase, 259
Noble Foundation, 233
Nocordia, 114
NO-dependent signalling systems, 106–8

nodulation, 227–8
  autoregulation, 211
nodules
  aluminium and, 226
  carbon restriction, 214–15
  determinate, 211–12
  indeterminate, 211–12
  infection zone, 212
  interzone, 212
  merismatic region, 212
  nitrogen feedback regulation, 217–19
  permeability to CO_2, 220
  permeability to O_2, 215–17
  phosphate deficiency, 227–32
    adenylate/P*i* levels, 228
    carbon metabolism, 229–31
    nodulation, 227–8
    oxygen diffusion, 231–2
  symbiotic nitrogen fixation in, 218*f*
Nostoc, 209
N-P ratios, 334
N-related morphological adaptations, 269–83
  developmental context, 274–5
  diagrams, 271*f*, 283*f*
  glutamate receptors, 279–80
  high C-N ratio-induced repression of lateral root initiation, 272, 278–9
  inhibitory effect of NO_3^- on lateral root elongation, 271, 273*f*, 277–8
  L-glutamate-induced regulation on root system, 272–4
  mechanisms, 275–80
  NO_3^- transporters, 280–81
  stimulatory effect of NO_3^- on lateral root elongation, 270–71, 272*f*, 275–7, 281–2
N-responsive genes, 42–6
NRT1 genes, 180
nrt1.1 mutant, 281
NRT2 genes, 180–81
*NtGln*1-5 gene, 7
NtGlt-3 gene, 7
NtOSAK, 158
nuclear magnetic resonance (NMR), 251
NUE (nitrogen use efficiency), 53–8
  definitions, 42*t*
  measurement, 24
  transcriptomics, 42–6
  variations, 54
NUpE (nitrogen uptake efficiency), 42*t*, 46–8, 47*f*, 54
NUtE (nitrogen utilization efficiency), 46–8, 47*f*
  canopy senescence, 50–53
  in crop plants, 49–53
  definition, 42*t*
  effect of nitrogen input and genotype interactions, 55*f*
  grain expression, 49–50
  sink processes, 49–50
  source processes, 50–53
  variations, 54
  in wheat, 57–8, 57*f*

O_2 diffusion coefficient, 215
o-acetylhomoserine, 174
oat, nitrate assimilation in, 69*f*
Oryza sativa, 317
OsNADH-GOGAT1 gene, 13
OsNADH-GOGAT2 gene, 13
Ostreococcus, 113
overexpression, 27
2-oxoglucarate, 190
oxosuccinamic acid, 20
oxygen diffusion, 231–2
ozone, 105

Paenibacillus, 114
Paracoccus denitrificans, 104*f*
Parasponia, 66
pea, nitrate assimilation in, 69*f*
peptide transporter (PTR), 180–81
perennial plants, nitrogen uptake in, 308
peribacteroid membrane (PBM), 89
Perilla fruticosa, nitrate assimilation in, 69*f*
peroxisomes, 161
peroxynitrite, 106, 137*f*, 149
Petunia, 254
pgm mutant, 257–8
Phaeodactylum tricornutum, 113
Pharbitis nil, 111
Phaseolus vulgaris, 3, 6–7, 12, 17–18, 186, 224*t*, 226, 229*t*, 332
Phenylalanine ammonia lyase (PAL), 154
phenypropanoid, 45, 255, 258, 324
Phleum pratense L., 334

phloroglucinol, 175
phosphate deficiency, 227–32
adenylate/P*i* levels, 228
carbon metabolism, 229–31
nodulation, 227–8
oxygen diffusion, 231–2
phosphates, 213
phosphoenolpyruvate carboxylase (PEPC), 214, 223, 230, 318
phosphoglycerate mutase, 43
phosphorus, in soil, 222
phosphorylation, 130
photon flux density, 337
photorespiration, 69–70, 293
up-regulation of pathway, 293
photosynthesis, 47*f*, 118*f*, 317–18
photosynthetic N use efficiency (PNUE), 335–6
phytochrome, 130
Phytophthora cryptogea, 140, 153
Phytophtora infestans, 140
P*i* levels, 231–2
Picea abies, 111
Pichia pastoris, 197
Pinus sylvestris, 19, 22
Pisum sativum L., 314
plant growth, 72–5, 306–11
plant metabolomics, 251–2
ammonia assimilation, 255–6
analytical platforms, 254–5
cross-talk between N and secondary metabolism, 258–60
isotopic labelling, 253–4
metabolic steady state and flux analysis, 252–4
N incorporation into primary metabolism, 256–8
nitrate assimilation, 255–6
plant nitrogen, 75–6
Plantago lanceolata, 332
plants
defence against herbivores and pathogens, 338–40
glutamate dehydrogenase in, 191–3
glutamate synthase in, 189–91
glutamine synthetase in, 185–6
leaf area index, 338
nitrogen acquisition by, 326–35
partitioning nitrogen into metabolites, 324–6
photosynthetic N use efficiency, 335–6
plant growth and nitrogen, 306–11
leaf growth, 315–20
photosynthesis, 317–18
seeds and generative growth, 321–3
shoot-root partitioning, 312–15
specific leaf area, 336–8
plasma membrane Ca^{2+} pump (PMCA), 151
plasmalemma, 255, 329
plastid transport, 95–6
plastidial phosphoglucomutase (pPGM), 257–8
Poa pratensis, 281, 337
polymerase chain reaction (PCR), 44
poppy plants, 253
Populus alba, 324
Populus deltoides, 335
Populus tremula, 308, 324
Priestly, Joseph, 104–5
primary metabolism, 256–7
programmed cell death (PCD), 159
Prokelisia marginata, 338
proline, 221
proline transporters (ProTs), 177
protein kinases, 155
PsAS1 gene, 23
PsASPG gene, 23
Pseudomonas aeruginosa, 177
Pseudomonas nitroreducens, 179
Pseudomonas stutzeri, 104*f*
Pseudomonas syringae, 140, 159
Pseudomonas taetrolens, 179
Puccinia triticina, 339–40
putrescine, 259, 259*f*
PvGln-α,7
PvGln-β,7
PvGln-γ,6
pyridine nucleotide, 294–5

quadrupole gas spectrometry (GC-QUAD), 251–2
quantitative trait loci (QTL), 24–5
Quercus faginea, 339
Quercus ilex, 339
Quercus serrata, 340

radiation use efficiency (RUE), 319
radish, nitrate assimilation in, 69*f*
reactive nitrogen species (RNS), 106, 158–9
reactive oxygen species (ROS), 106, 158–62
  candidate sites, 159–61
recombinant inbred line (RIL), 250
relative growth rates (RGRs), 308–9, 328, 335
reverse transcription polymerase chain reaction (RT-PCR), 260
RGRs (relative growth rates), 308–9, 328, 335
rhizobial bacteria, 66, 221
Rhizobium, 210–11
Rhizobium leguminosarum, 226
Rhizobium phaseoli, 224
rhizosphere, 72
Rhodococcus, 114–15
ribulose bisphosphate carboxylase activity, 324
rice
  GS genes, 7–8
  leaf area index, 317
  NADH-GOGAT gene3, 13
Ricinus communis, 316, 331
RIL (recombinant inbred line), 250
RNS (reactive nitrogen species), 106, 158–9
root zone, 72
roots
  ammonium uptake by, 87–8
  nitrate uptake by, 90
  N-related morphological adaptations, 269–83
    developmental context, 274–5
    diagrams, 271*f*, 283*f*
    glutamate receptors, 279–80
    high C-N ratio-induced repression of lateral root initiation, 272, 278–9
    inhibitory effect of NO_3^- on lateral root elongation, 271, 273*f*, 277–8
    L-glutamate-induced regulation on root system, 272–4
    mechanisms, 275–80
    NO_3^- transporters, 280–81
    stimulatory effect of NO_3^- on lateral root elongation, 270–71, 272*f*, 275–7, 281–2
ROS (reactive oxygen species), 106, 158–62
  candidate sites, 159–61
RUE (radiation use efficiency), 319
ryanodine receptors (RYRs), 151

Saccharomyces cerevisiae, 112, 183, 281
S-adenosyl methionine (SAM), 260
salicylic acid-induced protein kinase (SAIPK), 156
sarcoplasmic/endoreticulum Ca^{2+}-ATPase (SERCA), 151
Scenedesmus, 113
Schizachyrium scoparium, 338
Scirpus olneyi, 76
secondary metabolism, 258–60
seeds
  dormancy, 139
  generative growth and, 321–3
  germination, 139
seminal root length, 73*f*
senescence, 47*f*, 50–53, 140
senescence-associated genes (SAGs), 51
Septoria tritici, 339–40
serine, 256, 313
S-glutathionylation, 151
shoot-root partitioning, 312–15
shoot-root ratio, 296–7
signal transduction, 115–17
signalling, 147–63
  in animals, 148–52
    Ca^{2+} channels and transporters, 150–52
    metal nitrosylation, 148–9
    S-nitrosylation, 149
    tyrosine nitration, 149–50
  NO/ROS interaction, 158–62
    candidate sites, 159–61
    impacts on HR, 158–9
    protective molecule, 159–61
  in plants, 152–8
    Ca^{2+}-dependent protein kinase, 157
    Ca^{2+}-mobilizing messenger, 153
    intracellular Ca^{2+}, 152–3
    mechanisms, 153–4
    NO and protein kinases, 155
    NO/Ca^{2+} pathways, 154–5
    SNF1-related protein kinases, 158
signalling systems, 106–8
sink processes, 49–50

S-methyl-L-cysteine, 174
SNF1-related protein kinases (SnRKs), 158
S-nitrosylation, 149, 151
S-nitrothiols, 149
sodium nitroprusside (SNP), 137–8
soil acidity, 222–7
  aluminium and nodule development, 226
  aluminium and soil-borne rhizobia, 225–6
  aluminium and symbiotic nitrogen fixation, 223–5
  aluminium toxicity, 223
soil bacteria, 114–15
Solanum lycopersicum, 308
Solanum tuberosum, 307, 307*t*
Sorghum bicolor L., 317
soybean, genotypes, 217
Spartina alterniflora, 338
specific leaf area (SLA), 324, 336–8
specific leaf nitrogen (SLN), 318–21
spermidine, 259*f*
spermine, 259*f*
Spinacia oleracea L., 311
Sprengel-Liebeg Law of the Minimum, 333–4
steady-state metabolic profiling, 252–4
Stellaria media, nitrate assimilation in, 69*f*
stomata, 138–9
storage pools, 309–11
Streptomyces, 114
Streptomyces turgidiscabies, 114
Stylosanthes, 226
succinate, 259*f*
sucrose synthase, 214
sulphate assimilation, 118*f*
superoxide dismutase, 106
Symbiodinium bermudense, 113
Symbiodinium microadriaticum, 113
symbiotic nitrogen fixation (SNF), 209
  agricultural systems, 209–10, 209*f*
  aluminium and, 223–5
    nodule development, 226
    soil-borne rhizobia, 225–6
  aluminium toxicity, 223
  carbon restriction, 214–15
  drought stress, 213–22
    abscisic acid, 221
    bacteroid, 220–21
    leghaemoglobin, 219–20
  generalized scheme, 218*f*
  legumes in, 210–12
  natural systems, 209*f*
  nitrogen feedback regulation, 217–19
  nodule permeability to O_2, 215–17
  phosphate deficiency, 227–32
    adenylate/P*i* levels, 228
    carbon metabolism, 229–31
    nodulation, 227–8
    oxygen diffusion, 231–2
  soil acidity, 222–7
Synechococcus, 180
Synechocystis, 11, 112, 190

Taiyo Kagaku Co. Ltd, 197
tea, 173
  nitrogen fertilizer, 195
  theanine in, 173–5
  theanine metabolism, 175
  theanine metabolism in, 175
  theanine synthesis and transport in, 177–8
tetrahydrobiopterin (BH_4), 108
Tetranychus urticae, 338
theanine, 171–98
  ammonium transporters, 182–4
  chemical properties, 173
  chemical structure, 174*f*
  enzymes in synthesis of, 178–9
  gene expression, 196–8
  metabolism, 175, 196*f*
  nitrate transporters, 180–82
  nitrogen assimilation by GS-GOGAT, 184–5
  nitrogen uptake and transport, 179–80
  peak plasma concentrations, 171–2
  physiological benefits, 172–3
    anticancer properties, 173
    regulation of blood pressure, 173
  regulation, 193–5
    agronomic factors, 194–5
    genotypic factors, 193–4
  role in tea, 174–5
  site of sythesis, 177–8
  stimulatory effect, 172
  transport, 177–8
theanine hydrolase, 175–7
theanine synthase, 175–6

theta-brain waves, 172*f*
thigmotropism, 72
time-of-flight mass spectroscopy (TOF-MS), 251–2
Tradescantia, 138
transcriptome, 258
transcriptomics, 41–60
  crop production, 46–8
  gene expression, 53–8
  nitrogen utilization efficiency in crop plants, 49–53
    canopy senescence, 50–53
    grain expression, 49–50
  N-responsive genes, 42–6
transient receptor potential channels (TRPCs), 151
transport systems, 83–97
  ammonium transporters, 86–9
    in peribacteroid membrane, 89
    uptake by roots, 87–8
    vacuolar ammonia transport, 88–9
  Arabidopsis as model system, 86
  nitrate transporters, 89–95
    feedback and regulation, 92–3
    high-affinity transport, 91–2
    low-affinity transport, 90–91
    nitrate efflux, 94–5
    sensing role, 93
    uptake by roots, 90
    vacuolar, 93–4
  nitrogen forms available to plants, 83–4
  plastid transport, 95–6
  steps and mechanisms, 84–5, 85*f*
tricarboxylic acid (TCA) cycle, 211, 215, 254, 318
trifluoroperazine dihydrochloride (TFP), 157
Trifolium repens, 224*t*, 225, 229*t*
Trifolium subterraneum, 224, 224*t*
Triticum, 15
Triticum aestivum L., 315
tropane alkaloids, 258–9, 259*f*
tropisms, 72
tryptophan, 258
tyrosine hydroxylase, 150
tyrosine nitration, 149

ubiquitin, 27
ubiquitin ligase, 45
urea, 208
ureides, 213, 217
uric acid, 217
uridine monophosphate (UMP), 185*f*

vacuolar ammonia transport, 88–9
vacuolar nitrate transport, 93–4
vacuoles, 84
Venturia inaequalis, 339
Vicia faba, 153–4
vinblastine, 259
Vitis vinifera, 15

wheat
  canopy senescence, 50–53
  European varieties, 54
  gene expression and traits, 53–8
  grain expression in, 49–50
  leaf growth, 315
  nitrate assimilation, 70*f*
  nitrate uptake by roots, 328
  nitrogen management in, 47*f*
  nitrogen use efficiency, 53–8
white clover, nitrate assimilation in, 69*f*
white lupine, nitrate assimilation in, 69, 69*f*
Williams genotype, 217

xanthine dehydrogenase, 127
Xanthium sp., 69
Xerocomus badius, 173

Zea mays
  AtGC1 homologue, 111
  GS genes, 8
  leaf growth, 319
  nitrate assimilation in, 69*f*
  nitrate uptake by roots, 72–5, 329
  nitrogen and growth, 308
  nitrogen uptake and cycling, 330*f*
  transforming, 27
ZmGC1, 111
*ZmGln*1-3 genes, 2
*ZmGln*1-4 genes, 2